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REVIEW

BRACKEN AND THE GLASSMAKER'S ART

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Key words: Bracken, fern, glass, glass production, medieval, post-medieval, alkali, yield, seasonality, ash, historical documents.

ABSTRACT

Medieval and post-medieval documentary evidence records that glass could be manufactured using fern ash as a source of glass making alkali. This paper reviews the evidence for the use of bracken, the most abundant species of fern found in Europe, for glass manufacture by examining these early documentary sources and reviewing the contemporary archaeological evidence. Testing the viability of using bracken in glass manufacture by examining bracken growth patterns and yields, and its ability to form glass readily and produce a good quality product are reviewed and demonstrated through experimental investigation.

INTRODUCTION - BRACKEN IN GLASSMAKING.

Readers of this journal will be familiar with bracken, *Pteridium aquilinium* (L.) Kuhn, as one of the most commonly found species of fern found in Northern Europe. This plant has a history of use in diverse applications such as foods, medicine, soap making, fuel, litter, packing, bedding and flooring, and as roof thatch (Rymer, 1976). What may be less familiar is its use as a raw material for the glass industry from at least the thirteenth century (and possibly twelfth century (Polak, 1975)) throughout Europe. Whilst bracken (or related species) is common in many parts of the world, this is the first known reported use of this resource for glassmaking. Bracken contains significant quantities of alkali, most readily in the form of potassium compounds which act as a flux on the sand (Smedley *et al*., 1997).

At the transition from the medieval to the early Post-Medieval period there was an expansion of glassmaking throughout Europe and an increase in the quantity of glass, both luxury and utilitarian, is evident in the historical and archaeological record. It is also a time when there is clear experimentation with the use of different raw materials in glassmaking to furnish this demand (Cable, 2001a). In this context bracken would be a readily available, quick growing and an annually renewable resource. A detailed discussion of the use of bracken in medieval and post-medieval glassmaking has been the subject of three papers published since 2000 in Glass Technology and the context of this discussion and the findings of these papers will be summarised here (Smedley and Jackson, 2002, 2006; Jackson and Smedley, 2008).

Glassmaking in post-medieval Europe

Glass production in Europe from at least the 10th century until the advent of chemistry was a relatively simple process in terms of the raw materials used. Most glass was manufactured using a two-component recipe. The primary glass component was silica,

usually added in the form of a quartz sand, but sometimes as crushed quartz pebbles. The second component was a plant ash which contains high quantities of alkali, usually potassium or sodium, which acted as a flux. A flux was necessary as silica melts at temperatures in excess of 1700°C, which was too high for early furnaces; the potassium or sodium alkalis in the ash reacted by breaking the bonds in the silica to lower the temperature at which the silica starts to melt. Northern European glassmakers favoured plant ashes which were high in potassium as these were most readily available from hardwoods (e.g. beech or oak) or forest floor plants such as bracken. Southern European glassmakers used other plant ashes which were often more readily available to them than to the glassmakers of Northern Europe. These were plants which grew in salt-tolerant locations and so were higher in sodium. Often imported ashes were used as these were considered better quality and produced clearer (often 'water-clear') and better quality glasses. Soda also allows melting at lower temperatures than potash and so glasses would require less heat to form. It is noted that imported ashes were used for very high quality glasses produced in Venice (Jacoby, 1993, McCray, 1999).

The most detailed evidence of the use of specific plant ashes in early glasses comes from a number of surviving documents from the 12th century and onwards, which detail an extensive list of plant ashes which may have been used (e.g. Hawthorne and Smith (1979) on the writings of Theophilus; Merrifield (1967) on the writings of Eraclius; Smith and Gnudi (1990) on Biringuccio; Hoover and Hoover (1950) on Agricola; Cable (2001a) on the writings of Merrett and Neri; summaries of which can be found in Turner, 1956 and Jackson and Smedley, 2004). These include not only oak and beech, which are mentioned by many of these authors, but also reeds, wheat and barley straw, pea stalks and thistle, although it is unlikely these latter plants would make successful glasses.

EVIDENCE FOR THE USE OF BRACKEN

One of the plants repeatedly mentioned in many of these manuscripts is 'fern', although no species of fern is mentioned by name. As bracken is the most abundant species of fern found in Europe it is assumed by reviewers of these works, that it was the most commonly used fern in glassmaking (and the terms are often used interchangeably now as they were in the past (A.F. Dyer pers. comm.)). Supporting evidence for the use of fern or bracken can be found in contemporary documents such as glassmaking records or manuals, essays, bills of sale and parish records, in addition to more recent scientific analysis of early glasses and replication experiments.

Contemporary texts perhaps give us the most detailed and straightforward accounts of the use of bracken in glassmaking. The most compelling of these are those which detail the glassmaking process, although it must be noted that these are few in number and may be inaccurate as they often are written by observers rather than the glassmakers themselves (Moreland, 2001). The most detailed of these come from authors thought to originate from Southern Europe, most notably Italy, where high quality glass such as Venetian crystallo was manufactured. In these commentaries, fern is not used for the highest quality glasses such as those manufactured in Venice from imported soda-ashes, but for the manufacture of imitations in the venetian style (Façon de Venise) (McCray, 1999). The most well-known and arguably the most detailed of these are the manuscripts by Eraclius (Merrifield, 1967, probably written in the 12th century), Biringuccio (Smith and Gnudi 1990, manuscript published in the early 16th century) and Neri with later revisions by Merret (Cable, 2001a, original manuscript published in 1612). These manuscripts are thought to be of Italian origin, and each mentions the use of bracken in

a list of potential ashes used as glassmaking alkalis. Eraclius writes '*Glass is made with the ashes of .. fern'* and that the '*fern is cut before the feast of St John the Baptist, and well dried, and is then put into the fire and reduced to ashes'* (Merrifield, 1967: 212). Biringuccio refers to the importation of ashes made of fern from France '*First one takes some ashes made from the Herb salwort that comes from Syria; and I also understand ... that some comes from ... the Rhône in France. Now some say that this is made from fern and some from lichen ...*' (Smith & Gnudi, 1990: 127). All three authors discuss glass production using this ash in differing quantities, but Biringuccio and Neri also describe the production of a salt from the ash which was used to manufacture good quality clear glasses, in the style of the cristallo vessels manufactured in Venice. Biringuccio states '*another artificial salt is made that is called glass salt of sal alkali. By drying it the aforesaid salt for making glass is obtained.... The quantity desired by the workers is taken and boiling water poured on it; this makes very strong lye, and it gradually becomes thickened and clear by boiling. It is then dried so that it makes a very sharp salt ....'* (Smith & Gnudi, 1990: 127). A similar process is described by Neri (Cable, 2001a: 71). Jacoby (1993) also describes a 14th century Florentine recipe using fern ashes. There are records that fern ashes were used in glassmaking as late as the 18th century. Bosc D'Antic records his preparation of potash from bracken for use in the glassworks at Saint Gobain Glassworks, Paris (Cable, 2001b).

There are fewer references to use of ferns for glassmaking in Northern Europe and no mention in specific glassmaking manuals such as those by Theophilus, thought to be written in Germany in the 12th century (Hawthorne & Smith, 1979). That fern was used as an alkali in Northern Europe is evidenced by more anecdotal descriptions and indirect references. Rymer (1976) lists two texts which mention fern use in this context in the middle-ages. In the Squire's Tale (written around 1388), Chaucer states '*some seyden that it was Wonder to maken of fern asshen glass'* (Benson, 1988) and Norten in 1477 wrote that glass was made '*... of ashes of fern from the Lond'* (Rymer, 1976). Sir Thomas More writing in England in 1557 also pronounces '*Who wold wene it possible y glasses were made of ferne...*' (Newton, 1980). Other indirect sources for the use of fern in England come from bills of sale for the purchase of ashes, including green fern ash (dry fern ash is said to be not good) for the glass-house at Wollaton, Nottinghamshire in the 17th century (Smith, 1962). Likewise glassmakers purchased fern ashes in 1479 for the glass-house at Wolseley, Staffordshire, and there is evidence of individuals stealing fern without permission in the district, presumably for sale (Welsh, 1997; Smedley *et al.*, 2003).

Other less direct sources of evidence for the use of fern in glassmaking are the names of glass types, place names and legal documents. *Verre Fougere* (fern glass) was produced in France and other places, such as Bohemia, up to the 17th century. It is believed to have taken its name from the French district of Fougère (Savage, 1973). The Abbess of St Croix leased rights to gather fern to the glassworkers at La Ferrière and in return she received one gross of glasses (Knowles, 1927). The 15th century *Charte des Verriers*, a document which itemises the rights and privileges of noblemen in France also gives specific mention of the right to gather ferns for the preparation of alkali (Clark, 1931).

The written evidence presented above strongly suggests that bracken was used as a glassmaking alkali throughout Europe, both in its primary ashed state and as extracted purified salts prepared from the ash. However, material evidence linking bracken ash to glass production is more difficult to find. Archaeological evidence of ashes found on a

glassmaking site may just as well relate to the fuel ash waste as it does to the alkali used in glass production. Any ash used as an alkali would be a valuable commodity removed from the site once glassmaking had ceased. Thus it is difficult to show that fern ash, or indeed any specific ash, was used in the glass production process from archaeological remains.

The analysis of contemporary glass itself has not proved particularly successful in identifying specific ashes used in production. Chemical analyses have not yet clearly identified a chemical fingerprint for glasses thought to be produced using fern ash (see below). This is due to a number of reasons: (a) the variability between ashes of the same species from different sources (Turner, 1956; Hartmann, 1994; Jackson *et al.*, 2005), (b) because ashes themselves can be mixed before use in glass production masking any fern ash 'fingerprint' and (c) glass melts can be supplemented by the addition of cullet, which is the recycling of ready formed glass from various sources (such as the material collected from bottle-banks today). The addition of cullet is advantageous for glassmelting as it reduces the temperature at which the raw materials melt, but it dilutes the initial composition and contaminates the glass, making the identification of the initial raw materials very difficult. However, there has been some tentative initial success in the identification of glasses produced with fern ashes as a generic group compared to those produced using hard woods such as beech and oak (Jackson *et al.*, 2005 and see later). Both Tyson (2000) and Henkes (1994) suggest that glass makers from the 12-15th and the 17th century respectively used fern ashes west of the Rhine (except Alsace) and produced 'forest glasses' with hardwood alkalis, such as oak and beech, to the east of the Rhine. Glassmakers in the Netherlands used fern ashes in an attempt to produce cristallo, a fine, high quality clear glass produced in Italy (Henkes, 1994).

The review of evidence for the use of fern in glassmaking throughout Europe from the 12th century onwards suggests that fern, or bracken, was a satisfactory source of glassmaking alkali and was used throughout Europe. However, its seasonal behaviour, low yield per hectare compared to hardwoods and labour intensive harvesting regimes might suggest that its widespread use was not as common as these sources might suggest and that it may have been used to supplement other ashes, or for specialist forms of glassware. Consequently, the use of fern ash for glass production has been dismissed by some modern authors (Stern and Gerber, 2004). To investigate whether bracken is a good raw material to use for glass making, bracken yields, its seasonality and harvesting times, and its melting ability and product quality are examined below.

BRACKEN AS A GLASSMAKING RESOURCE

Seasonality

In contrast to hardwood plant ashes used for glass production which could potentially be harvested throughout the year, bracken is a seasonal plant and can only be harvested through the growing season. Furthermore, it grows in stands or colonies which progressively age to the extent that the central zone becomes less active, leading to uneven growth distributions (Atkinson, 1986; Watt, 1976). Therefore changes in yields and availability can occur over time within a particular location. The transfer of energy resources between different parts of the plant during the growing season can also be accompanied by changes in the chemistry of the plant during the growth cycle. However, its ability to grow in a wide range of habitats and colonise easily may nevertheless make it an attractive plant for glassmakers.

The easy availability of bracken, which grows in different environments and across

large areas of land, might suggest that this raw material was more commonly available than hard woods, and so influence the location of the glass-house. However, a more costly resource, which was needed in greater quantities, was hardwood for fuel. As glass production required high temperatures and long firing regimes wood was consumed in great quantities. Therefore although the availability of bracken ash would influence the location of the glass-house, the need for readily available fuel was much more important. Consequently glasshouses, before the use of coal for fuel, tended to be located in woodland which could be harvested for fuel. This is apparent throughout Europe, and specifically in Britain, where medieval and early post-medieval production was located in the Weald, Stourbridge and North Yorkshire. Thus, whilst glasshouse location was driven by the need for fuel, usefully these same areas often were ones which yielded sufficient bracken for use in glassmaking.

The factors influencing yield and re-growth patterns were investigated in an experimental study of the sustainability and yields of bracken for glassmaking by Smedley and Jackson (2006) using bracken collected from two geographically different sites through a single year and from a single site which had been repeatedly harvested. The first site, Snake Pass, Derbyshire, (GR SK131895) is located on a steep south-facing hillside at an altitude of 280m above sea level, with a bedrock of millstone grit and a soil chemistry low in nitrates of pH 5.6. The second site, Rugeley, Staffordshire (GR SK997180) is located on slightly undulating terrain at 175m above sea level, within a clearing, but surrounded by dense birch and pine woodland, with an underlying geology of kuyper marl and sandstone, a high nitrate soil with a more acidic pH at 4.5. The average mean temperature at Snake Pass is lower by around 2°C, and the average rainfall approximately 20% higher, than at Rugeley. Changes in chemical composition of bracken

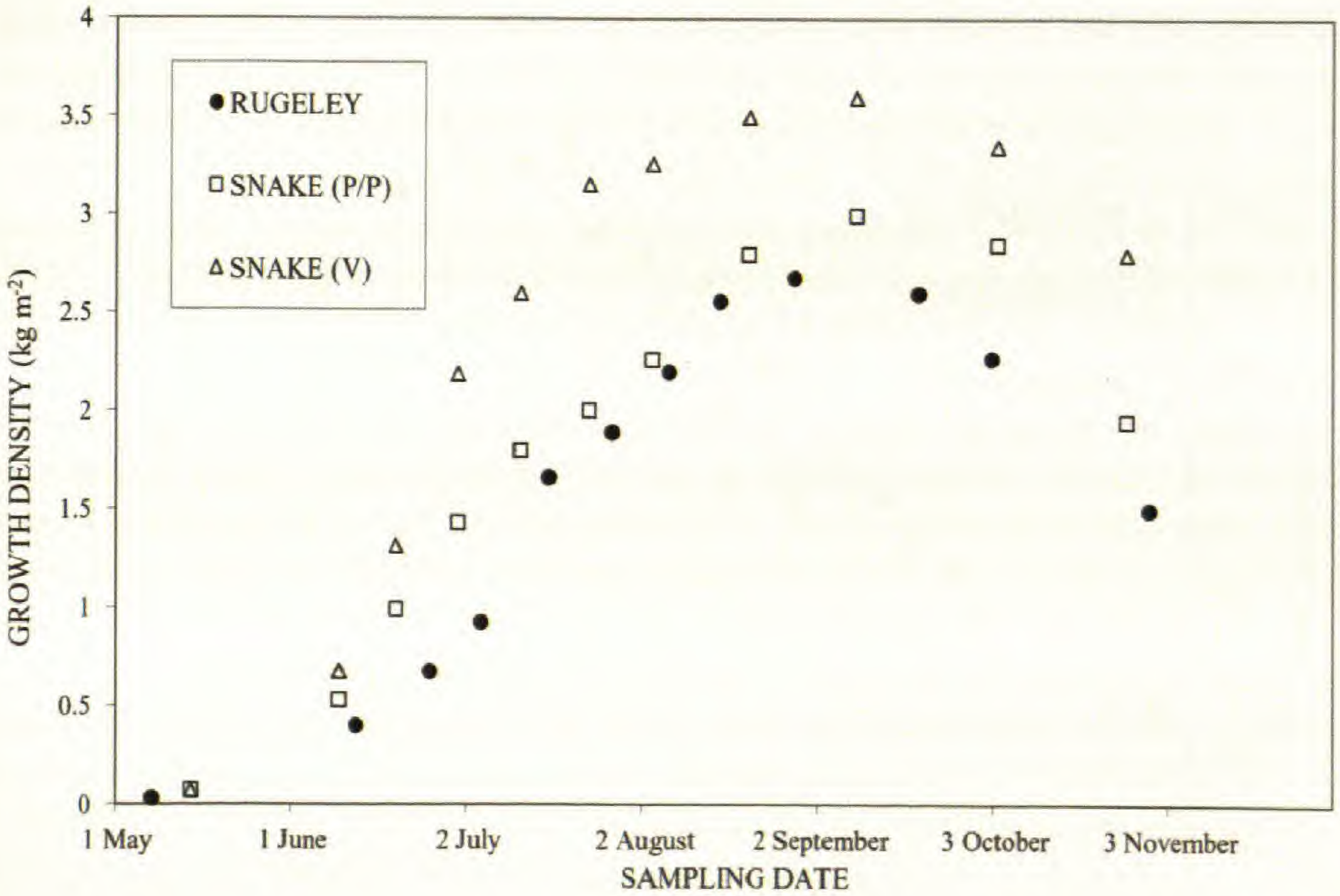

Figure 1. Weight of green bracken per unit area collected from the sites of Rugeley in Staffordshire and the Peak District (Snake Pass) from an area which had not been picked previously (v) and that which had been repeatedly harvested (pp).

at these two sites through the growing season, detailed below, are documented in Jackson and Smedley (2008).

The results relating to growth patterns at the two sites suggest location, aspect and soils have a significant effect upon growth habits. Frond density (Figure 1), weight of green bracken collected per unit area and final weight of ash per unit area collected (Figure 2) were higher at the higher altitude hillside site of Snake Pass. Whilst the growing season is slightly shorter here than at Rugeley, the aspect, increased rainfall and nitrogen-richer soils may have favoured a higher yield. Repeated harvesting at Snake Pass was shown to significantly decrease the yield of ash (Figures 1 and 2), presumably due to a decrease in the ground litter which would offer protection to bracken at the start of the growing season and the depletion in stored nutrients in the rhizomes once the bracken had started to die back in the autumn (Lowday, 1986). Bracken, which was continually harvested near glass-houses (the complex of structures which contained the glass furnaces and associated equipment) in order to reduce transport costs of this raw material, would gradually deplete in yield. One way to reduce this would be to employ a rotational harvesting strategy in order to maintain yields but keep transport costs low; the high costs associated with harvesting along with increased transport costs if bracken (presumably already ashed to reduce volume) were brought some distance to the glass-house would make this an uneconomic raw material.

The results relating to growth patterns would have been of interest to the medieval bracken collector in terms of supply locations and potential yields. However, contemporary documents relating to glassmaking, from the 16th century onwards, suggest there was an optimum time to harvest bracken for glass production. This in turn suggests that harvesting did not take place throughout the growing season, but perhaps only once at each location and the time of harvesting was particularly important for glass production. Southern European authors are quite specific when this should take place; Eraclius says that bracken was traditionally cut around the time of the Festival of St.

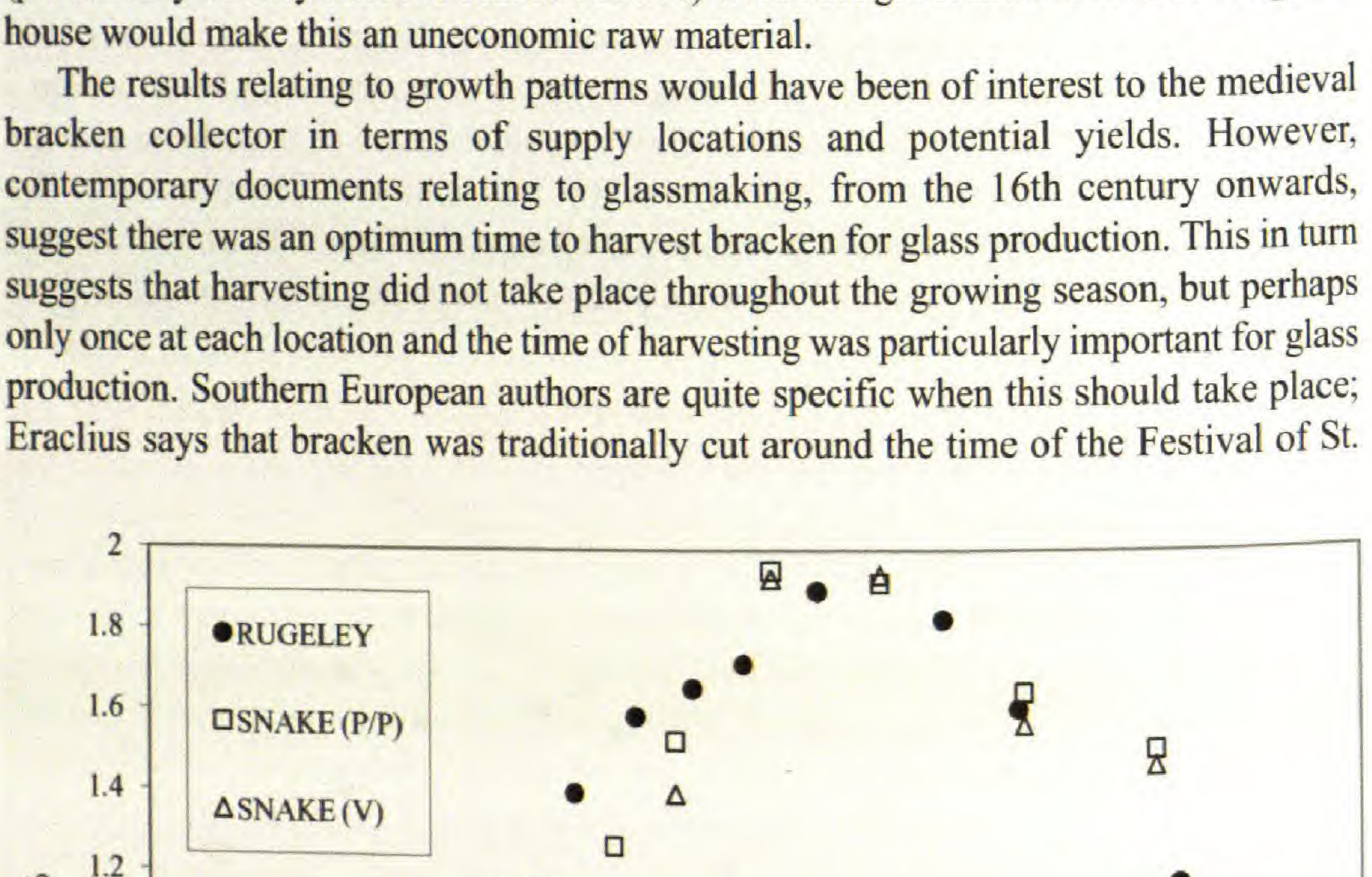

Figure 2. Weight of bracken ash per unit area from the three sites in the study area (Rugeley, Snake Pass (v) and Snake Pass (pp).

John the Baptist (24th June; Merrifield, 1967: 212). Neri suggests ferns were harvested from the end of May to mid-June and Merrett notes this is when the plants are in full growth (Cable, 2001a: 71). For Northern Europe the dates are less exact. Cutting bracken for any purpose in the 16th century was forbidden before 1st August (the reasons for this are unclear) (Polak, 1975: 12) and a document by Fosbrook, sourced by Smith (1962), notes that 17th-century glassmakers at Wollaton in Nottinghamshire use '*green fearne asse*' but that '*Dry fern asse is not good'*, again a comment which suggests a harvest before the late summer. These latter references suggest that glassmakers used bracken harvested at the peak of its growth. The experimental data presented in Figures 1 and 2 show that in the UK, bracken is at the height of its growth cycle and produces the highest yield of ashes in late August/early September. The result from the experimental work does not coincide with the dates for harvesting in May suggested by Eraclius and Neri, although Merrett does suggest this is when fern is in 'full growth', which might be in May/June in southern Europe, but later in Northern Europe. The experimental data show that the yields are highest in the study area much later in the year, and as the bracken collector would want to maximise his profit (greater weight per unit area/decreased time to collect sufficient for sale), this might suggest a late harvest and even later glass production cycle for English glassmaking as the harvested crop would need to be dried and ashed before use.

It is thought that glass-houses were in operation, producing glass, for only part of the year, often when the weather was more favourable, certainly in northern climates. Polak, (1975: 15) suggests that in 13th century Venetian glass-houses were active from January to August, in Germany from Easter to November. If the bracken ash was only available towards the end of the season it might be suggested that the ash collected in one season was used for glass production in the next.

Yield

The yields of ash from green bracken at both sites and both harvesting regimes were in the order of 2% ash to green weight. Whilst this seems very low in modern terms, these results are similar to the ash yields from hardwoods which are thought to be more commonly used in glass production (Jackson *et al.,* 2000; 2005; Smedley *et al.,* 1997; 2003). So, once harvested and dried (which would take less time for bracken than for hardwoods) the associated yields would be similar for both alkali raw materials.

Maximising the yield of the raw material and thus reducing the costs of the alkali is only one aspect of glassmaking. If the raw material costs are low but (a) the quality of the resulting glass is poor (most particularly in terms of a strong colour), (b) or the amount of fuel needed to melt it is high because the raw materials do not melt easily and higher temperatures are needed, then it is unlikely the glassmaker would use these raw materials. As it is doubtful that more than one harvest of bracken at each location would be made, the glassmaking capabilities of the ashes at the point of harvest is particularly important. Further experimental work, undertaken by Jackson and Smedley (2008), explored changes in bracken composition through the growing season which would influence the melting behaviour and the quality of the resulting glass, and in turn influence the time bracken was harvested for glass production.

Melting ability, glass forming and glass quality

Hunter (1953) examined some major and trace element constituents of bracken through the growing season. He reported that nitrogen, phosphorus, potassium and magnesium

concentrations decreased with the age of the frond, but calcium and sodium contents increased (Hunter, 1953: 20). While nitrogen, and phosphorus in low concentrations, are of relatively little importance, potassium, magnesium, calcium and sodium are important for glass melting (Turner, 1956). Also of importance is silica, which can be found in considerable quantities in plant material. The alkalis, potassium and sodium, act as fluxes. These elements are found in relatively high quantities in plant ashes. (To a more limited extent phosphorus can act as a flux, along with iron, although these are not the primary fluxes and are usually found in lower quantities in glasses.) Magnesium and calcium are important for final glass stability (they stabilise the glass so that the alkalis are not lost by leaching over time), but can have a detrimental effect on the melting behaviour of the glass. Calcium (and magnesium) in excess can make the glass more difficult to melt and therefore the glassmaker requires a higher temperature to produce a clear fully melted glass. However, high quantities of either or both in the final glass conversely make the glass less stable. Silica is the main glass former (it forms the basic glass molecular structure), and has a very high melting point. Too much silica in the ash again reduces the alkali proportionately and increases the total silica in the glass making it more difficult to melt, except at very high temperatures (Jackson & Smedley, 2004).

The results of compositional studies of bracken by Jackson and Smedley (2008) are in general agreement with those of Hunter (1953). They also discuss in more detail some additional elements which are important to glass melting or quality. Figures 3 and 4 show that the concentration of silica (SiO_2), alumina (Al_2O_3), calcium oxide (CaO) and soda (Na_2O) rise in the fronds through the growing season. All of these components, with the exception of soda, produce a less reactive ash. However, soda is in such low concentrations (below 1%) that its increase in concentration through the growing season will not be particularly significant for glass melting (Figure 4). Potash (K_2O) concentrations fall through the growing season. Both sodium and potassium alkalis are important as fluxes so their combined concentration in the ash would affect melting behaviour; soda is also a more effective flux than potassium, although in this case the increase in concentration through the season would not compensate for the decrease in potash concentrations. Iron and manganese are elements which would impart strong colours to the glass. Iron produces a green or blue hue depending upon the furnace atmosphere and manganese, in excess, a purple hue. These two elements also increase through the growing season (Figure 5). It is presumed that the glassmaker would prefer glasses which were not highly coloured. At relatively high concentrations for colourants, for example above 0.5wt% in each case, the combined elements would produce a rather dark brown, almost-opaque, glass.

Looking at these compositional results together, the combined elemental patterns throughout the growing season suggest that it would be preferential to the reactivity of the ash, and hence ease of melting, and the final clarity and colour of the glass, to use ashes from bracken which was harvested earlier in the season.

Bringing the two competing factors of yield and melting ability/glass quality together the best time to harvest the bracken to reduce the refractory and colouring components, increase the alkali fluxes to make the ash most reactive and have sufficient yield to keep costs low, would probably be in early June. The earlier harvest of bracken than that proposed from yields alone would optimise the drying time and the harvester could take advantage of the warmer and drier conditions of the summer months, possibly reducing the need for covered storage before ashing. This observation corresponds with that reported by Neri and Eraclius. The later date, after the 1st August reported by Polak

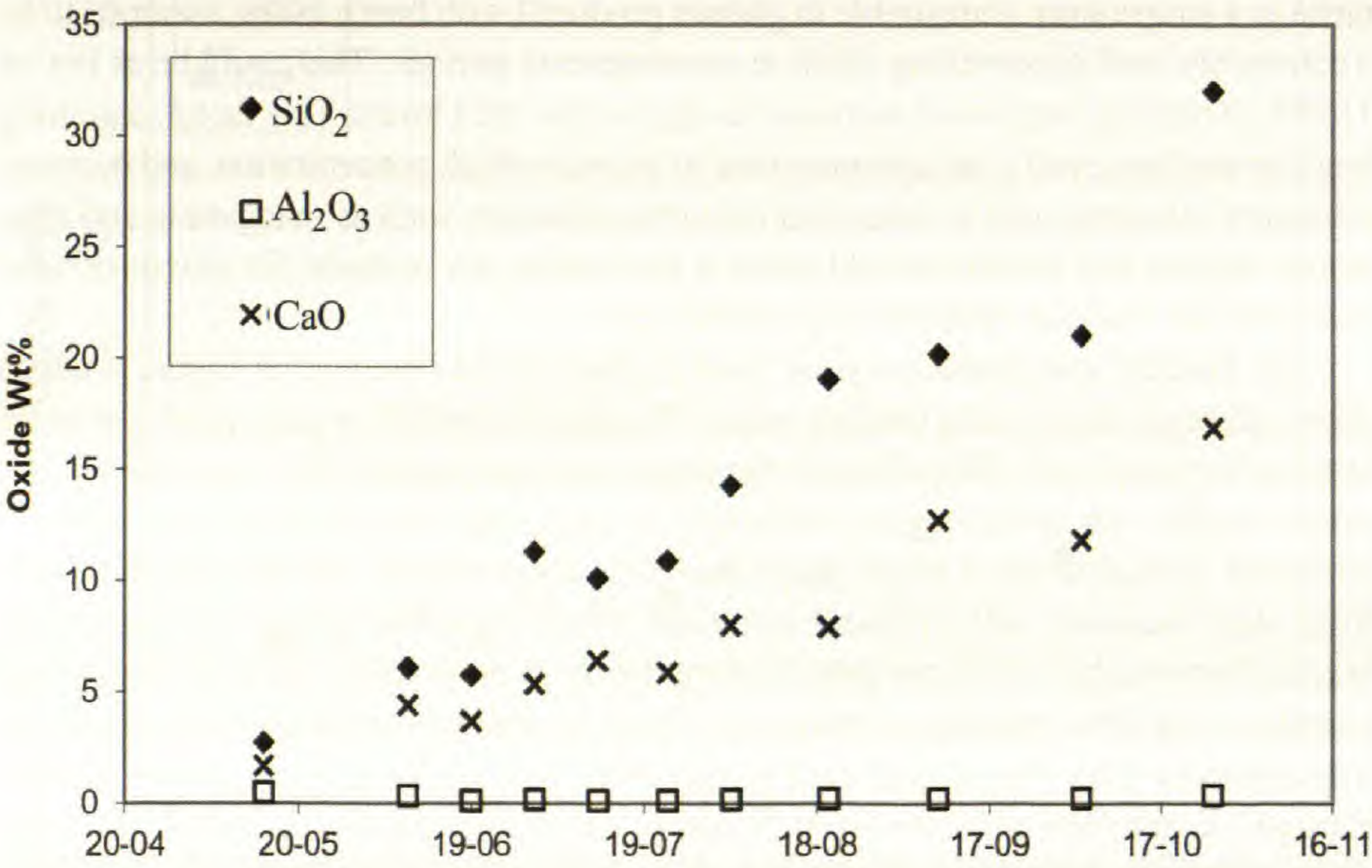

Figure 3. Concentrations of SiO_2, CaO and Al_2O_3 in bracken fronds through the growing season (Snake Pass).

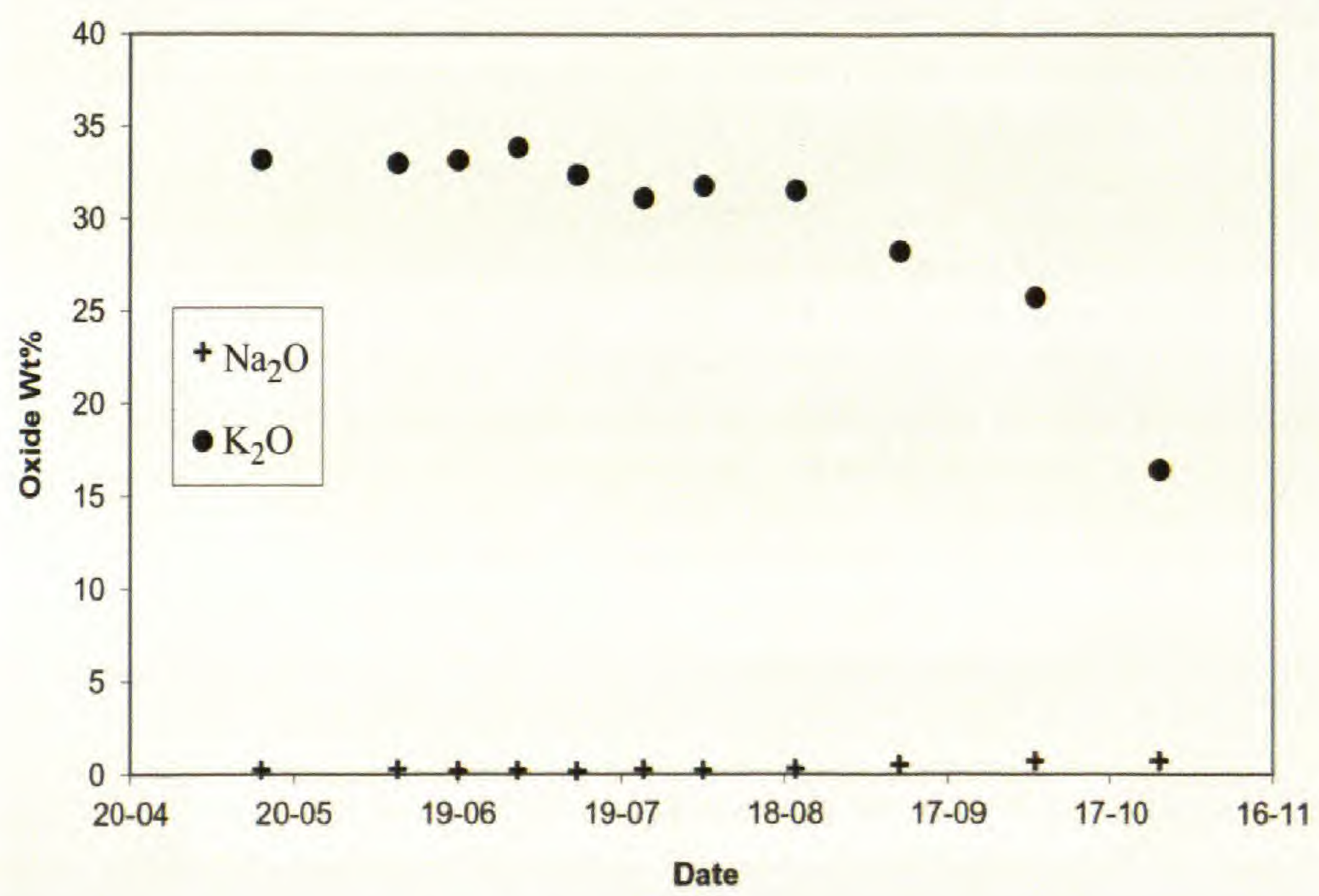

Figure 4. Concentrations of the alkalis, Na_2O and K_2O, in bracken fronds through the growing season (Snake Pass).

(1975:12), may be for the use of bracken for purposes other than glassmaking.

Jackson *et al.* (2004) report that glass made with bracken collected in early summer melts at a temperature comparable to glasses produced with beech ashes, assumed to be a commonly used glassmaking alkali in contemporary periods. This could be as low as 1100ºC if melting times were increased to days rather than hours. This factor, assuming bracken was harvested at an optimum time to increase alkali concentration, and decrease refractory elements such as silica and colouring elements such as manganese and iron, would suggest that bracken would prove a favourable raw material for glassmaking if sufficient ash could be obtained at a suitable cost.

That bracken can produce a good quality glass can be observed in Figure 6 which shows glass produced using bracken ashes. The glass on the left is glass produced using bracken harvested early (May/June) in the season and pure sands (L30A Loch Aline silica sands used in the modern glass industry); a relatively clear lightly-tinted glass is produced. This glass has a slight purple hue. Glasses produced with bracken harvested from other locations with different soil chemistries, or indeed glasses produced with bracken harvested at a different point in the growing cycle, could be more strongly tinted purple (owing to the presence of manganese) or even tinted green (from iron). If salts are manufactured from the ash and used as the alkali flux (Jackson *et al.*, 2000), as both Biringuccio and Neri advocate for high quality, colourless glasses, the results are more spectacular and a clear glass, almost crystal in quality, is produced (Jackson *et al.*, 2000; Figure 6 right).

HISTORIC BRACKEN GLASSES

There are few reported examples of glass furnace sites where it is thought that bracken was used as the primary alkali source. Welch (1997) suggested glass was manufactured with local bracken ashes at the 14th to 16th century glassmaking site at Little Birches, Staffordshire, although this is based upon bills of sale. Crossley (1967) has speculated that fern ashes may have been used at the glassmaking site of Bagot's Park also in Staffordshire in the 16th century from the analysis of the glass found there, and again in Yorkshire (Crossley & Aberg, 1972). Other authors have also used chemical analysis of glasses to suggest that fern ashes were used for glass production from the medieval period in France (Barrera & Velde, 1989), Germany (Gerth *et al.*, 1998; Hartman, 1994; Wedepohl, 1997), Belgium (Terlinden & Crossley, 1967) and Britain (Mortimer, 1997; Tennant *et al.*, 1984; Sanderson *et al.*, 1984). These studies have been variable in their degree of success in the identification of the specific ash, partly because of the known heterogeneity of plant ashes within and between species and by growing environment (Turner, 1956; Sanderson & Hunter, 1981; Hartmann, 1994; Jackson & Smedley, 2004; Jackson *et al.*, 2005).

However, at this time a rather general degree of identification of the type of plant ash used in historic glasses can be postulated. Using the low number of analyses of bracken and other ashes the pattern emerging is that, whilst hardwood ashes cannot easily be differentiated to species, there appears to be some distinction between fern (bracken) ashes and glasses produced from them and glasses produced using hardwood ashes. Bracken ashes and bracken ash glasses contain a higher proportion of potash (K_2O) to lime (CaO), often in the ration of 3:1 or more, whilst hardwood ashes often contain equal or lower proportions of potash to lime. The analyses of more samples of bracken ashes from different locations may help to elucidate this, taking into account the difficulties

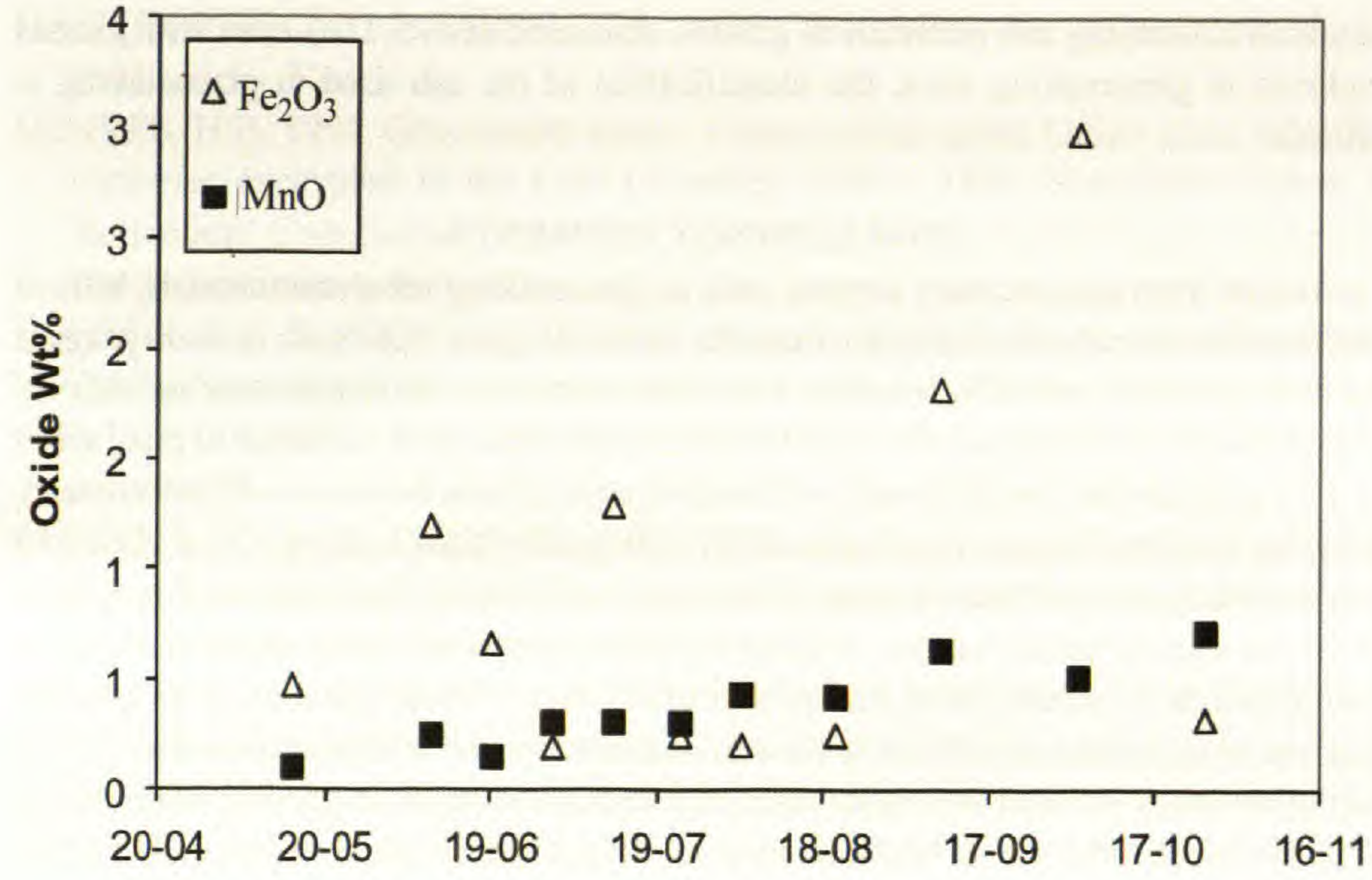

Figure 5. Concentrations of the main elements responsible for colour in the glass, Fe_2O_3 and MnO, in bracken fronds through the growing season (Snake Pass).

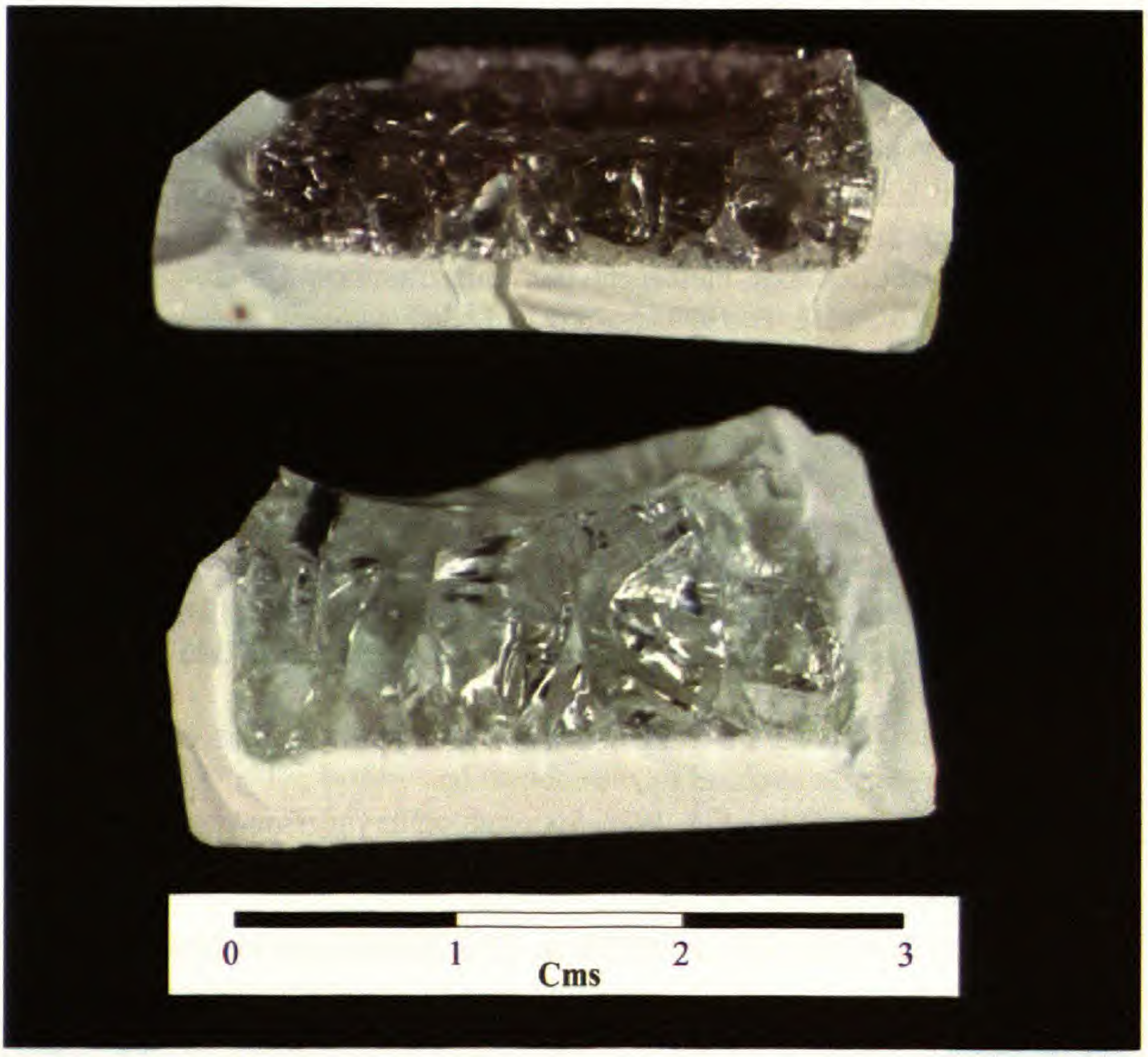

Figure 6. Model glasses produced using bracken ash and Loch Aline Sand (top) and bracken salts and Loch Aline Sand (bottom).

presented identifying raw materials in glasses, discussed above. Thus even with glasses produced at glassmaking sites, the identification of the ash used in glassmaking is difficult.

CONCLUDING COMMENTS

It is known from documentary sources such as glassmaking texts and manuals, bills of sale, legal documents and indirectly from the names of glass types such as *verre fougere* that ferns, probably primarily bracken, were used extensively in the glassmaking industry from at least the 13th century. Bracken however, was only one of a number of plant ashes used for glass production. In Southern Europe the ashes from the eastern Mediterranean, including Syria and Egypt were imported for high quality glass making. Other glasses in both southern and northern Europe were made with ashes from seaweed and from hardwood such as beech and oak. It is reported that many other plant species have been used although it is difficult to accept that all of these would produce good glasses. Bracken, by its nature, is difficult to harvest in quantities and so it may have been mixed with other ashes, or used in a more restricted fashion to produce good or high quality glass. However, there are sufficient sources to suggest that its use was not limited either geographically or in scale. In the wider context of historical glass production bracken plays a novel and important role; today it is widely considered an invasive and undesirable plant with little or no economic value. However up to the more recent past bracken was considered a valuable economic resource for a variety of industries, not least for its role in good quality glass production.

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SHORT NOTE

OREOGRAMMITIS SETOSA AND *O. SINOHIRTELLA* IN THAILAND

Four species of *Oreogrammitis* Copel., a grammitid genus of Polypodiaceae, are reported for Thailand on the Ferns of Thailand website (address below), revised 1 Sept. 2014. Two of these, *O. congener* (Blume) Parris and *O. reinwardtii* (Blume) Parris, are names that have been changed from the Grammitidaceae account in The Flora of Thailand Vol. 3 Part 4 (Tagawa & Iwatsuki, 1989), where they are identified as *Grammitis setosa* Blume and *G. bongorensis* [error for *bongoensis*] (Copel.) Copel. respectively.

Tagawa & Iwatsuki (1989) describe the stipe hairs of *Oreogrammitis setosa* (Blume) Parris (syn. *Grammitis setosa*) as being up to 1 mm long. Material from Chanthaburi province (*Iwatsuki & Fukuoka T.7156,* E!) has stipe hairs up to 1.5 mm long and undoubtedly belongs to *O. setosa*; it is a good match for material from Java, where the type of the species was collected. *Oreogrammitis congener* has stipe hairs 0.1-0.3(-0.5) mm long, while *O. setosa* has stipe hairs (0.1-)0.5-2.0(-2.5) mm long. It is possible that *O. congener* occurs in Thailand, but I have seen no material.

Tagawa & Iwatsuki (1989) note that *Oreogrammitis bongoensis* (syn. *Grammitis bongoensis*) is the only species of *Grammitis* in North-eastern Thailand, and that its habit on moist mossy rocks is distinctive. *Oreogrammitis bongoensis* is endemic to Borneo and is distinct from the Northeast Thailand material (*Tagawa, Iwatsuki & Fukuoka T.1825*, E!), which belongs to the recently described lithophyte *O. sinohirtella* Parris, also known from China and Vietnam. *Oreogrammitis reinwardtii* differs from both *O. bongoensis* and *O. sinohirtella* in having the lateral veins slightly prominent to prominent on both surfaces of the lamina, while they are not prominent on either surface in *O. sinohirtella*, and either not prominent on either surface or sometimes slightly prominent only on the adaxial surface in *O. bongoensis*. *Oreogrammitis bongoensis* has stipe hairs (0.9-)1.6-2.7(3.3) mm long and laminar margin hairs (1.9-)2.0-2.8(-3.4) mm long, while *O. sinohirtella* has stipe hairs (0.2-)0.4-1.2(-1.8) mm long and laminar margin hairs (0.1-)0.3-1.9(-3.0) mm long. It is also possible that *O. reinwardtii* occurs in Thailand, but I have seen no material.

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B. S. Parris

MONITORING CHANGE IN *ISOETES HISTRIX* BORY (ISOETACEAE) AT ITS NORTHERN DISTRIBUTIONAL LIMIT

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Key words: *Isoetes*, Quillwort, Cornwall, conservation, survey

ABSTRACT

A re-survey of the threatened Land Quillwort (*Isoetes histrix* Bory) at its northernmost range extent on the Lizard peninsula, Cornwall, indicates a massive decline (>90%) in total population size since 1982 with the loss of several major sub-populations. The factors responsible for this are considered and climatic changes, particularly in the pattern of rainfall, and management practices, such as the reduction in grazing, are implicated in its decline.

INTRODUCTION

The genus *Isoetes* is a cosmopolitan one, comprising about 80 species of specialised heterosporous lycopods growing predominantly as submerged aquatics, although some species occur only as terrestrials in seasonally inundated habitats. *Isoetes histrix* Bory (Land Quillwort) is a small summer-deciduous perennial, growing from a 'corm'. Its specific name derives from the rather spinose persistent leaf bases which are typically but not invariably produced and whose function, whether purely protective, or perhaps to aid in dispersal, is open to conjecture. It is a plant of open, often trampled, bare shallow soils, frequently overlying rock, which are wet and preferably flooded in winter, but dry in summer. Throughout its range it occurs over a wide variety of underlying rock types, although in its British mainland sites, the most northerly by some 200 km, it is restricted virtually to serpentine, just one site occurring on schists and another at the serpentine/schist junction (Byfield & Pearman, 1999). A Mediterranean-Atlantic species, it is found from Cornwall, Guernsey and Alderney in the British Isles, down through the Atlantic coasts of France, Spain and Portugal, and along the NW African coast almost to Tunisia. In the Mediterranean it occurs on many of the islands, e.g. Rumsey *et al.* (2001), and is scattered as far east as the west coast of Turkey (Jermy, 1965). There is a map of its total range in Bolòs & Vigo (1984).

ISOETES HISTRIX IN THE BRITISH ISLES – A BRIEF HISTORY

The Land Quillwort was first discovered in the British Isles in Guernsey "in damp spots on Lancresse Common in June 1860" (Wolsey, 1861). Marquand (1901) added Cobo and Petit Port as localities on the authority of Derrick, while further documenting the areas on L'Ancresse where the species could be found. McClintock (1975) neatly summarised the history of the species on Guernsey – "it was collected at Cobo in 1910-13; towards La Rochelle in 1928; and in 1957 was detected on Hommet Paradis and Hommet Benest, and on Lihou, where at least two patches are known. Albecq was added in 1969". He noted that it had not been re-found near Petit Port, at the Guet, nor in its

most inland station, a marshy field between Vale School and L'Ancresse, where it had been collected in 1906. Its stronghold was still at L'Ancresse where it was then known in at least five places from Fort Doyle to the La Varde area. Ryan (1990) knew of nine populations on the north and west coasts and the immediate offshore islands, with a total population size in 1988 estimated at 10,670 plants. The greatest part of this was however to be found in an area of just 17 x 1.5 metres. More recently Ozanne (2005) lists the plant as occurring in six sites, three of them offshore islets: Lihou, Hommet Paradis and Hommet Benest. It was not refound at the last by Gibby *et al*. (1997), who found the species only on the Fort Hommet headland. The species was discovered by Marquand (1902) on Alderney. He reported it as "abundant at the top of the cliffs to the east of La Quoire, over a space of about 100 yards, and on the slopes below". Bonnard (1996) records it in two contiguous 1 km squares at the eastern end of the island. The total population size in 1988 was estimated at 30 or so plants (Ryan, 1990).

It was not discovered in Britain until June 19th 1919 when it was found at Caerthillian, on the Lizard peninsula (Robinson, 1919). He gathered the plant with the Lizard speciality clovers *Trifolium strictum* L. and *T. bocconei* Savi and noted "I saw one specimen only, but am convinced it is probably to be found in many similar situations. The specimen is small and the plant would be very difficult to find unless especially looked for". While initially accepted by Druce (1920), who noted "microscopical examination shows that it is undoubtedly the same as the Guernsey one", he obviously later had doubts, stating in the Comital Flora "The Lizard record is an error" (Druce, 1932). It was not properly confirmed until re-found in 1937 (Melville, 1938). Melville, like Robinson before him, initially found only one plant while looking for other rarities but unlike Robinson he had the opportunity to revisit the site the following day and extend his search. While conceding that recognition was difficult because of the similarity in form to other taxa with curled grass-like leaves, he reported "once the type of habitat favoured by the plant and the identity of its usual associates had been discovered, it was a relatively simple matter to explore the neighbourhood and determine its distribution". He went on to establish its range to be "from a little to the south of Vellan Head southwards along the coast to a point south of the Lion rock above Pentreath beach, and reaches inland for half to three quarters of a mile". A month later N.Y. Sandwith extended the known range a short distance to the north-west of Lizard village refinding it in Robinson's locality in the Carthillian [sic] Valley (Melville, 1938). Plants were collected from Melville's direction by I. Manton in 1938 and 1939 for cytological investigation (Manton, 1950) and remained in cultivation at Manchester University into the 1950s. John Raven found it at Gew Graze and Mullion in 1950, Clive Jermy near Black Head in 1963, and then we know of a string of records from David Coombe from the late 1960s. There are very few other known records before the commencement of the University of Bristol Lizard project (UBLP) in the 1970s, but by the time of Margetts & David (1981) it was known from 12 stations in SW61 and SW71. It was first detected in SW72, in a shallow quarry north of Traboe Cross, in 1984 (Murphy *et al*., 2012).

John Hopkins, who was part of the UBLP team and did his PhD on the Lizard from 1977 onwards, found many more sites, but taking into account that there was no real knowledge of population numbers, it was decided to survey every known site in April 1982, and the results of this survey were published in Frost *et al*. (1982), including 1:10,000 maps with each site and its population marked thereon. This detailed report also gives much background information on the habitats in which the plant occurred and presented recommendations for its conservation.

The UBLP team visited (or noted where they were unable to gain access) 68 'sites', where a site was defined as a population clearly separated from another, even if only by a few yards. They estimated a total of nearly 98,000 plants, stressing that even that might be a conservative estimate.

Like so many plant projects, once a definitive study has been carried out, we all relax, and assume that all is done and all is well. Certainly there were no systematic surveys carried out for nearly 30 years, until the current authors commenced their recording in 2010, though there were some notable new sites found by Rosemary FitzGerald in 1999.

CONSERVATION STATUS IN THE BRITISH ISLES

Perring and Farrell (1977) in the first edition of the British Red Data Book regarded the species as Rare but did not consider it to be at threat, because of its inconspicuous nature and early season appearance. This view was not shared by Frost *et al.* (1982) who identified several potential threats. By the third edition of the Red Data Book the species was considered to be Near Threatened (Byfield & Pearman, 1999), some sites having known to have been lost to fire, cultivation and the lack of grazing, although most recorded sites were still extant and the most recent census counts (i.e. Frost *et al.* (1982)) indicated a large total population. Cheffings & Farrell (2005) raised its conservation status to VU D2 on the basis of its very restricted range, with the evidence cited above of at least limited decline which it was believed was ongoing. Anecdotally changes in land-use and conservation management on the Lizard peninsula since the last major survey of its botanical specialities had suggested major declines since the 1980s in a suite of species of open and disturbed habitats such as the distinctive track-ways upon which many Quillwort sites had existed. A detailed re-survey was clearly imperative given the very patchy knowledge of its current distribution and abundance, an impetus driven in part by the desire to make a more accurate assessment for the forthcoming England Vascular–Plant Red-list, but also to properly assess the efficacy of recent changes in conservation management on the Lizard peninsula.

MATERIALS AND METHODS

In the four years commencing 2010 all of the sites identified in the Frost *et al.*, (1982) report, and all subsequently discovered sites (see above), along with others found during our survey – a total 85 sites, have been visited during the species peak growing season (October – May) at least once and in many cases repeatedly.

In 1982 the UBLP adopted two techniques for counting. In areas that were sufficiently small a direct count was made; in larger sites the total area in which the plant occurred was measured and then a 5% sample of the total area was taken using randomly sited 1×1m quadrats. These were counted and then totals for the whole were extrapolated. It is not known how many populations were assessed by this latter method, although it certainly applied to the huge population at Predannack (SW6715).

All of our counts are direct, rounded up to the nearest 10. We too had trouble assessing the Predannack site but felt confident in giving a maximum possible total based on the direct counts without recourse to extrapolation from a randomised sub-set as used previously.

RESULTS

Since 2010 we have visited all the UBLP sites, often in each of the four years, together with the 17 'new' sites found since the 1980s and during our survey, 85 sites in total.

Our 'success' rate has been most disappointing, though each year we imagine that climatic factors have reduced the likelihood of discovery in that particular year. The summary of results is given in Table 1, where we have found, since 2010, plants at 37 sites, but only at 20 out of the 68 sites (30%) recorded in the 1982 survey. The numbers counted, though not fully using their methodology (if only because the numbers this time are so low), are seemingly less than 3% of those found then. The extent and distribution of these losses at a 1 km scale is visualised in Figure 1.

Smaller (mostly <100 plant) marginal populations in four 1 km squares (SW6814, SW6913, SW7116, SW7217) have been lost. However, this has been offset to an extent by the discovery of one major new site in SW7113, where we have reported up to a thousand plants, and the species' continuing presence in the most northerly and isolated inland site, found originally in 1984, in another hectad at SW7321. We consider these sites to have previously been overlooked rather than being genuinely new, i.e. formed post 1982.

What is not particularly apparent even when the distribution is given at 1 km scale are the major losses of sub-populations within sites witnessed since the 1982 survey (Figure 2). This is particularly evident around Mullion (SW 6617) where the plant had been widely scattered, associated chiefly with the network of moderately used tracks dissecting this area. Since 1982 the plant has apparently been lost from 15 of the 18 documented locations (83%) with a decrease in plant numbers of >97%.

DISCUSSION

Frost *et al.* (1982) acknowledge that 1982 was a particularly favourable year to yield data on the maximum size that the Lizard population was likely to reach under the then present conditions. The major drought of 1976 and the consequent fires had killed much of the vegetation that might compete with *Isoetes*, and the report suggested that the consequent bare ground may well have been colonised by sporelings, which by 1982 might have grown into mature and luxuriant plants and thus were more easily detectable by eye. In addition the winter of 1981/82 was wet and mild. In the four years of our survey three of the winters have had cold spells, and that for 2012/2013 was very mild and wet until a very cold March (apparently the coldest for 100 years) and April with easterly winds. It is therefore with some caution that we consider the apparent massive decline in the species abundance.

The results of our re-survey pose many questions, many of which remain unresolved and will form the basis for ongoing study.

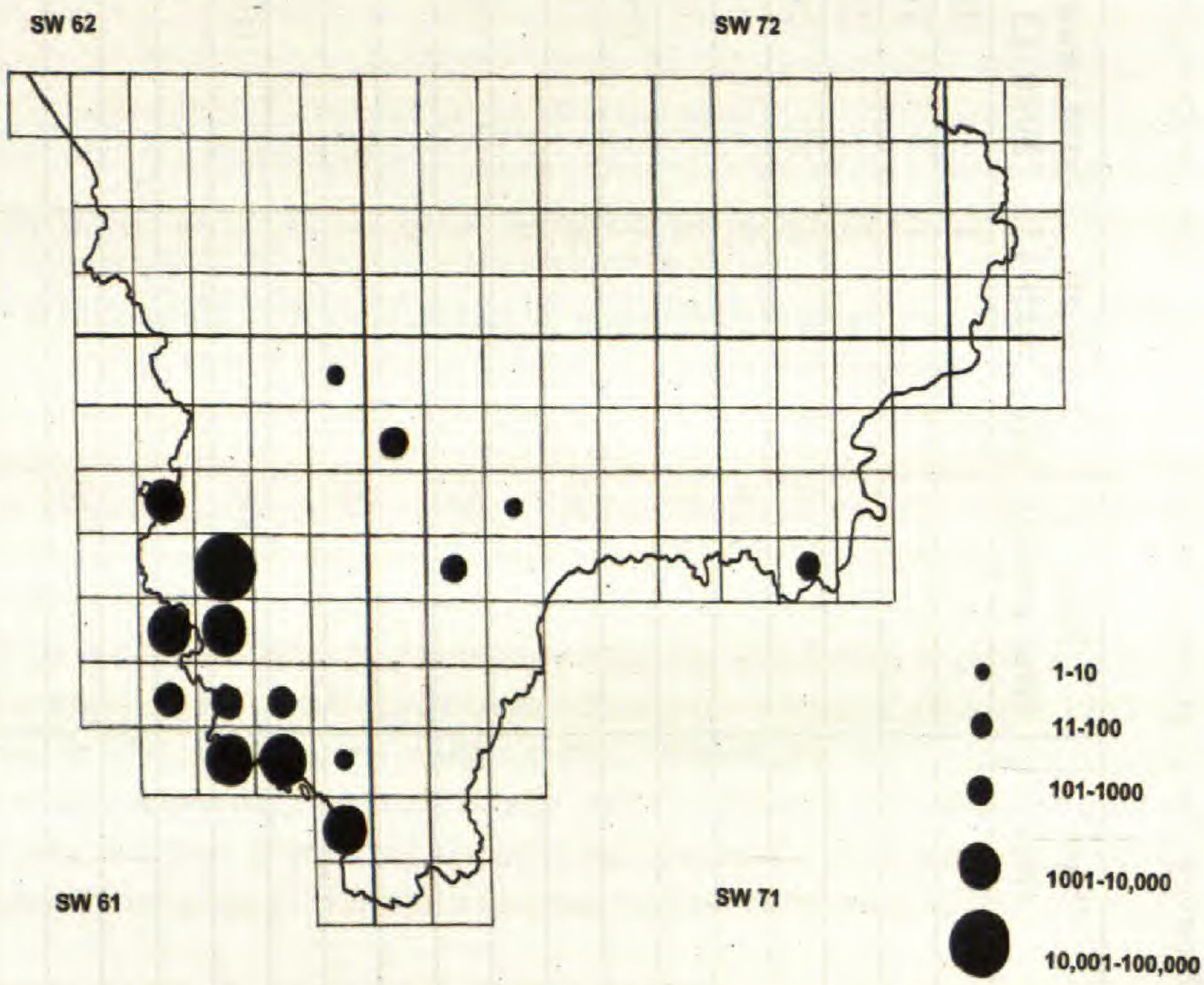
1. Has the vegetation become higher or denser, and if so, does this have an effect on sporelings or even mature plants?

Whilst there has been much clearing of scrub in the last few years, and, by and large, adequate levels of grazing, we do not really know if there was a period since 1982 of sustained or substantial under-grazing which might have smothered the plants. Certainly the single biggest site on the mound at Predannack, where the UBLP counted the astonishing total of 68,998 plants in 1982, did go through a period of scrub encroachment, and we have never counted more than a few 100s (possibly +/- 1,000 if we had been more systematic).

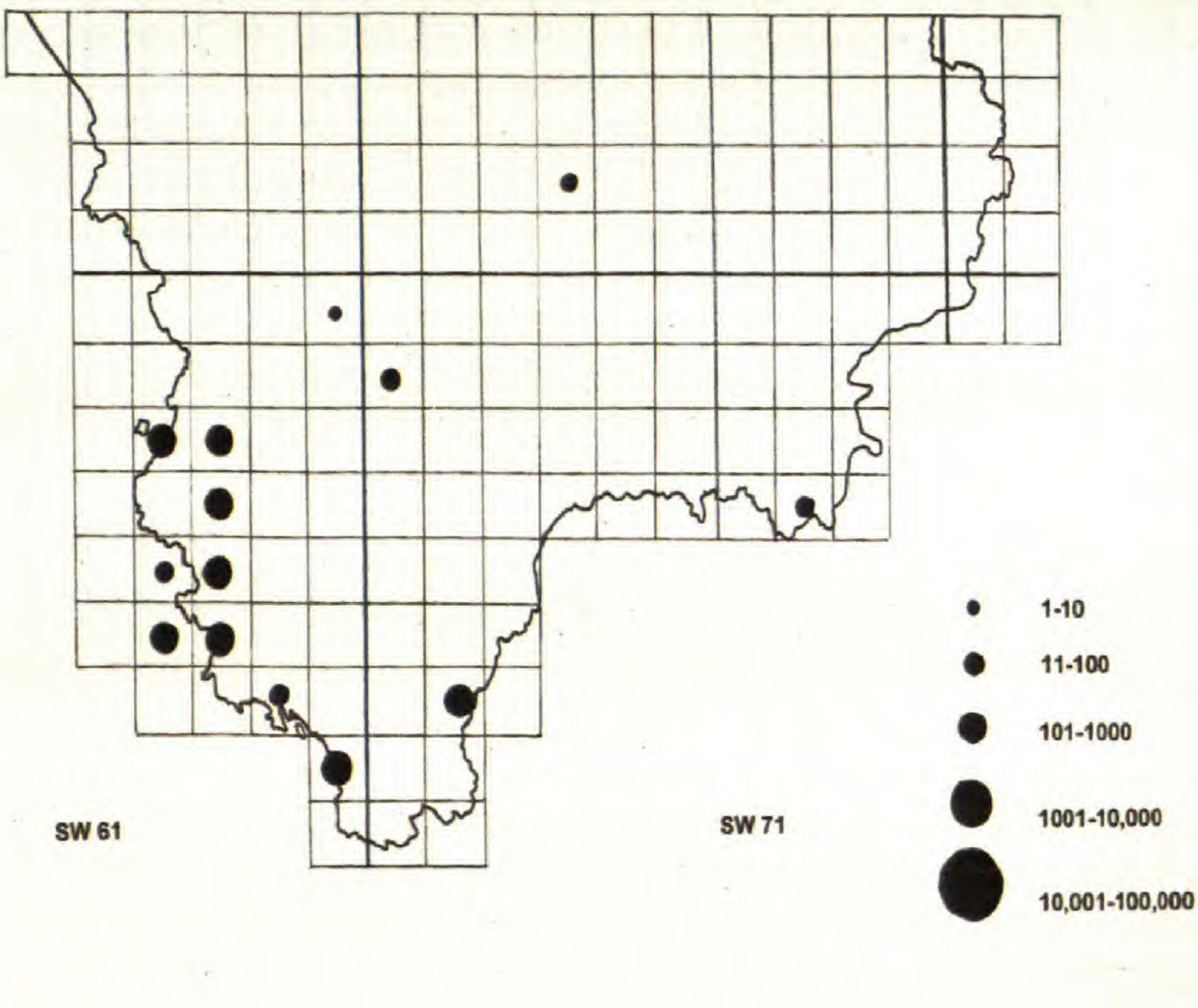
The UBLP report did say that *Isoetes* grows, *inter alia*, in shallow soils with a covering of dwarf herb-rich turf, but is there a point when the turf becomes too high? Density of cover seems to matter less. The account in the Red Data Book (Byfield &

Table 1. Summary of site and population counts of *Isoetes histrix* at the Lizard within 1 km squares (there were small addition errors in the 1982 totals). The figures in the 2013 columns only relate to the same sites as in the 1982 survey. The population totals for the 'new' sites are the highest counted in any of the years 2010 – 2013.

1 km sq. SW	Sites 1982	1982 counts	Sites 2010-13	Counts 2010-13	'New' sites	'New' sites counts	Total all sites 2010-13	Total counts 2010-13
6614	4	550	1	300			1	300
6615	1	1650	1	100			1	100
6617	18	9000	3	200			3	200
6713	5	2460	3	250	3	10	6	260
6714	6	650	1	300	4	220	5	520
6715	4	1050	0	0	2	120	2	120
6715	1	69000	1	1000			1	1000
6813	10	6700	3	30	3	50	6	80
6814	2	170	0	0			0	0
6912	8	6500	2	400	2	300	4	700
6913	1	0	0	0			0	0
6919	1	0	0	0	1	10	1	10
7018	1	60	1	30			1	30
7113	0	0	0	0	1	1000	1	1000
7116	1	20	1	20			1	20
7217	1	0	0	0			0	0
7321	0	0	0	0	1	20	1	20
7716	4	80	3	50			3	50
Totals	**68**	**97890**	**20**	**2680**	**17**	**1730**	**37**	**4410**



A



B

Figure 1. Distribution and abundance of *Isoetes histrix* on the Lizard peninsular in A: 1982 and B: 2010-2013 mapped at a 1 km scale.

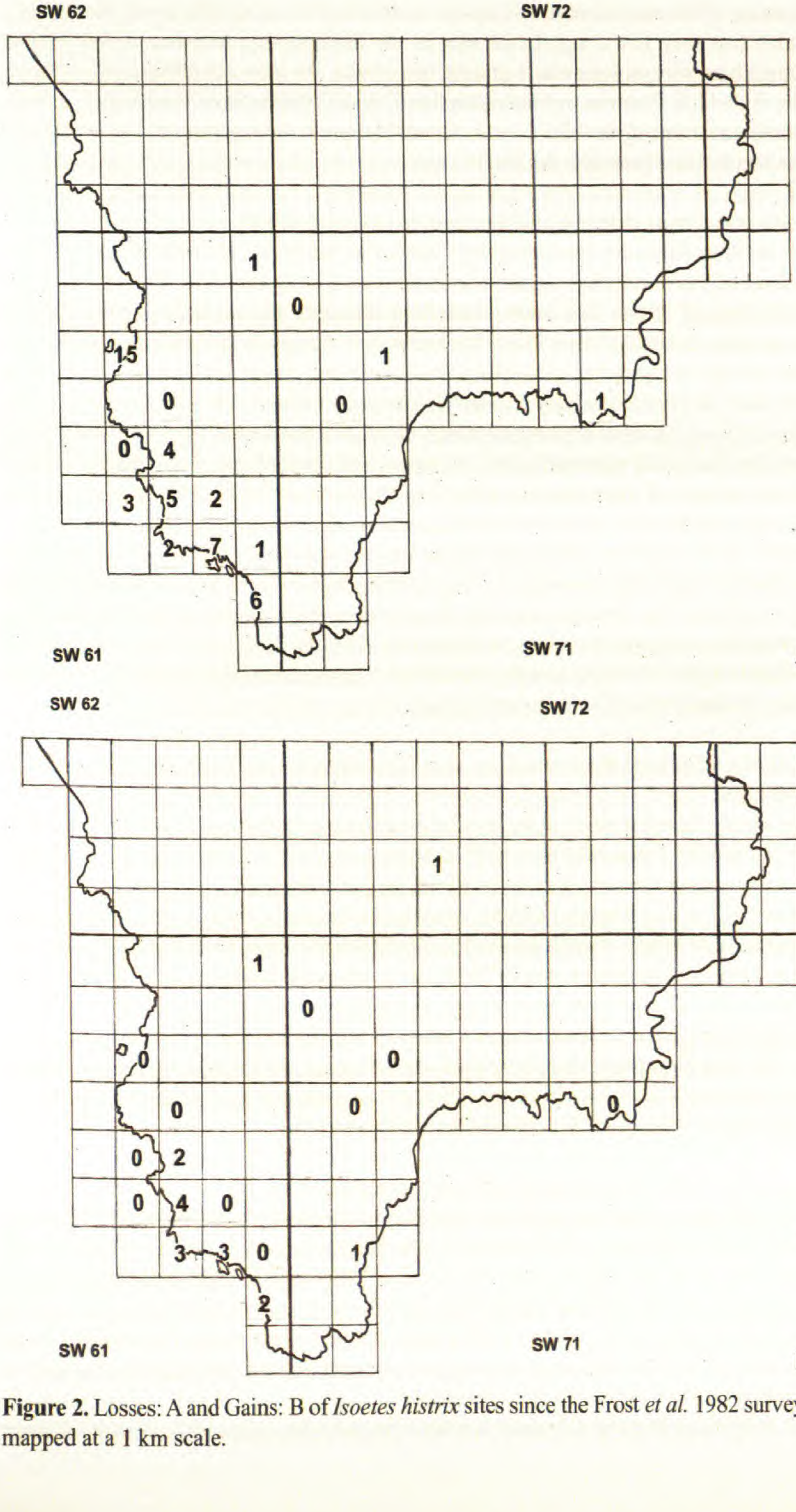


Figure 2. Losses: A and Gains: B of *Isoetes histrix* sites since the Frost *et al.* 1982 survey, mapped at a 1 km scale.

Pearman 1999) stated that it will survive in short turf at up to 80% cover, though they added that they felt it significant that in the 1982 survey, four out of five extinct populations were on sites where grazing had ceased. We have not refound any of those. The Byfield & Pearman account adds that David Coombe, from Cambridge, a long-standing member of the UBLP team, found that spores have germinated in wetted soil samples that had been kept dry for 34 years.

2. Has the climate changed, and if so, has that had any effect?

Recent years have seen very dry springs and wet summers and autumns. We do not know if this is having an effect on the Mediterranean species, but Ilya McLean from the University of Exeter has been researching this with his students and wonders if temperature is not the major driver but rather that changes in moisture availability are. Interestingly we have had very little success in finding plants before March, other than one site with consistently early records (Holestrow); whereas in the UBLP report they cite 'leaves emerging in late September or early October depending on the season and usually with the first heavy autumnal rains'. It appears that research has shown that the pattern of dry springs and wet autumns is not giving the species of Mediterranean pools such as *Cicendia filiformis* (L.) Delarbre a chance to adapt. Species such as *Minuartia verna* (L.) Hiern. (at its southern limit) and *Isoetes* were 'losers' and others such as *Trifolium scabrum* L. and *Scilla autumnalis* L. were adapting better. Certainly our experience is that the vegetation has become very lush by autumn, which would militate against bare ground for seeding or sporeling establishment. Andy Byfield (pers. comm.) wonders if it could be that *Isoetes* is actually short lived – more annual than perennial – and that spore production is curtailed by dry springs.

3. On the other hand there must have been dry springs before, though possibly not such a sequence?

We wonder then if the loss of the population of *Isoetes* on the Kynance slope (SW6813, where the UBLP found 5408 in 1982 and where we cannot find a single plant, despite looking a dozen times at all times in the winter and spring) has become more droughted since 1982, is thus a natural decline, whereas the species is doing well in places with a more reliable supply of moisture (such as the Holestrow area). However the slopes must have been very droughted after 1975 and 1976, but perhaps this created a suitably open area for the spores to colonise (perhaps producing a temporary population explosion) dying away again once wetter seasons returned, with the closing of the grassy canopy?

We should, perhaps, categorise each site by aspect and slope, as well as by habitat (predominately around rock outcrops, but also on paths and in coastal erosion pans), in order to see if that gives any clue to survival.

4. Has increased visitor pressure affected the populations?

The 1982 survey does suggest that light tourist trampling is favourable to the plant, particularly as the bulk of this occurs in the dormant season – June to September. We think that there are more tourists, and we also think that the visiting season is longer. The major site for path populations was at Mullion, where we have seen only small to medium numbers in two places, with smaller numbers along Pengersick (SW6714) and the Rills (SW6713), which we have largely re-found. But the decline in the numbers at these path sites is no greater than elsewhere.

Away from the paths we cannot see that increased visitor pressure has had the slightest

effect – indeed surely adjacent bits that were formerly rather rank become better suited for *Isoetes* because of extra trampling?

5. Have sites for other plants been lost in the same period?
Over the last five years we have only looked in detail at the rare plants, and amongst those, we have concentrated almost entirely on the annuals. The number of extant sites for the two rare *Juncus* species– *J. capitatus* Weigel and *J. pygmaeus* Rich. ex Thuill. and the numbers in the remaining sites, seem to have dwindled alarmingly, and similar results have been found for two of the rare clovers – *Trifolium bocconei* and *T. strictum* (the third, *T. incarnatum* L. subsp. *molinerii* (Balb. ex Hornem.) Ces. seems fine, though never in the numbers counted by the UBLP in 1977 (Frost *et al.* 1982). Similarly there have been reductions in a suite of bryophytes associated with the *Isoetes,* such as the small winter-annual thallose hepatics of the genus *Riccia*, e.g. *R. bifurca* Hoffm. and *R. nigrella* D.C. and the leafy hepatic *Gongylanthus ericetorum* (Raddi) Nees (all completely or largely restricted to the Lizard peninsular in the British Isles) (Holyoak, 2010). It must be born in mind that these results are based on only five year's surveys, though we have looked at every site every year. To us, at least, each season has seemed to have had something wrong with it, and certainly the lush summer growth, as mentioned above, has meant less bare ground for seedling germination.

6. Is there any evidence of decline in other parts of its range?
A detailed re-survey of the Channel Islands populations is also wanting, but Gibby *et al.* (1997) and more recent researches by Fred Rumsey (pers. comm.) strongly suggest a similar decline in both sites and population size in Guernsey and the only available counts in Alderney (Ryan, 1990) would suggest a decline from "abundant" in 1902 to c. 30 plants in 1988. Further south in mainland Europe we have only looked at Flora Iberica, where there is no comment, and the Flora of Finistère (Quéré *et al.*, 2008). In the latter they say that the species was looked for in all its historical stations (along with *Ophioglossum lusitanicum* L., a regular associate there): the map shows only one 10 km square that has not been updated. They do not mention losses, but the maps are only at 10 km² scale.

We are planning a meeting in April 2014 to look again at every site, past and present, and aiming to use the same methodology as the 1982 survey. We still know so little about its reproduction and individual plant longevities, its dormancy potential and other ecological traits that, coupled with its apparent dramatic decline, we feel that ongoing monitoring is essential.

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IN VITRO PROPAGATION AND CRYOPRESERVATION OF THE ENDANGERED FILMY FERN, *TRICHOMANES PUNCTATUM* SUBSP. *FLORIDANUM* (HYMENOPHYLLACEAE)

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Key words: *Trichomanes punctatum* subsp. *floridanum*, cryopreservation, endangered, gametophyte, *in vitro*, propagation, sporophyte

ABSTRACT

In vitro methods were investigated as a tool for propagating and preserving tissues of the endangered filmy fern, *Trichomanes punctatum* subsp. *floridanum*. Wild-collected sporophytes were grown *ex situ* in soil and were used as a source of material for establishing in vitro cultures. Gametophytes, germinated from spores, were grown and propagated on agar plates, but were not successfully surface sterilized for in vitro growth. Sporophyte cultures were successfully cryopreserved using the encapsulation-dehydration method and showed good survival through both drying and freezing. Tissues up to 24 months old survived cryopreservation, although the percentage of tissue pieces surviving was lower than with younger tissues. Older tissues also had lower levels of total soluble carbohydrates. Pre-culture of sporophytes for two days on ABA did not affect survival compared with pre-culture on medium lacking ABA. However, younger tissues lacking any pre-culture showed reduced survival through cryopreservation compared with pre-cultured tissues. These results demonstrate that *in vitro* propagation methods could be used to provide plants for restoration, while cryopreservation could play a role in the *ex situ* conservation of *T. punctatum* subsp. *floridanum*.

INTRODUCTION

Trichomanes punctatum Poir. subsp. *floridanum* W. Boer (Florida bristle fern) is a rare filmy fern (Hymenophyllaceae) that is listed as endangered in Florida and is a candidate for federal listing as threatened or endangered. While other subspecies are found in the Caribbean and tropical America, *T. punctatum* subsp. *floridanum* is endemic to Florida, with fewer than 1000 plants in five occurrences (U.S. Fish & Wildlife Service, 2012).

The plant was first discovered in Miami-Dade Co. in south Florida in 1906, growing in limestone sinkholes or solution holes in rockland hammocks and later, in 1936, in a similar habitat known as "fern grottoes" in Sumter Co. in central Florida. It is threatened by habitat loss, invasive species, as well as by drainage for agriculture that has lowered the water table in the south Florida area. *Trichomanes punctatum* subsp. *floridanum*, as well as other plants growing in the sinkhole/solution hole habitat, relies on underlying water to maintain an environment of high humidity (Nauman, 1986; U.S. Fish & Wildlife Service, 2012).

Because of these threats, *ex situ* propagation of the species could be useful in

providing a back-up to wild populations and in providing plants for augmentation and re-introduction. *In vitro* propagation has proven successful with a number of pteridophytes (e.g. Camloh and Ambrožič-Dolinšek, 2011; Pence, 2004), and thus, the following experiments were undertaken to investigate the potential of *in vitro* methods and cryopreservation for assisting in the *ex situ* conservation and restoration of this species.

MATERIALS AND METHODS

Sporophyte Propagation

Sporophyte tissues of *Trichomanes punctatum* subsp. *floridanum* collected in Miami-Dade Co., Florida, were received at the Center for Conservation and Research of Endangered Wildlife (CREW) from Marie Selby Botanical Garden, in June, 2005 and in October, 2005. Tissues were rinsed with pure water and put into soil boxes - clear plastic boxes with lids (Sigma Phytatrays), which were filled to about 3 cm in depth with soil-less potting mix (ProMix) that had been sterilized by autoclaving at 250°C and 18 psi for 45 min.

Sporophytes were initiated into culture using two methods of surface sterilization. 1) Tissues were surface sterilized in a 1:20 dilution of commercial bleach for 5 minutes with stirring and rinsed in sterile, pure water. 2) Other tissues were sterilized using a 1% solution of sodium dichloroisocyanurate (DCIC, Aldrich) for 5 minutes, followed by a rinse in sterile, pure water. After surface sterilization, tissues were cultured on Murashige and Skoog salts with minimal organics (Linsmaier and Skoog, 1965) (MS medium) at 1/2- or 1/4-strength, with 1.5% or no sucrose and gelled with 0.33% Gelzan (Caisson), with the addition of 100 mg/L benlate (methyl 1-(butylcarbamoyl)-2-benzimidazole carbamate, Aldrich) (Ben) and one drop (approximately 0.05 ml) of a solution of antibiotics as previously described (Pence, 2005). Once established, the sporophyte cultures were maintained on half-strength MS medium with 1.5% sucrose plus 0.33% Gelzan (½ MS medium). Cultures were grown either in 60 x 15 mm disposable petri plates or in 25 x 150 mm borosilicate culture tubes with caps (Magenta™ two-way), with 15 ml of medium per plate or tube. Cultures were incubated at 26°C with a 16:8 light:dark cycle, under CoolWhite fluorescent bulbs, at approximately 20 $\mu E/m^2/s$. For acclimatization, plants were removed from culture, rinsed of any excess medium using reverse osmosis (RO) water, and planted in soil boxes, as used for the initial growth of sporophytes (above).

Gametophyte Propagation

One collected plant had sori and the spores were cultured separately on 0.8% agar (Sigma A1296) plus 100 mg/L Ben (agar + Ben) in 60 x 15 mm disposable petri dishes, and germinated into gametophytes. Several attempts were made to initiate sterile cultures of gametophytes using 0.01, 0.1, and 1% DCIC. Gametophytes, along with some associated agar were surface sterilized for 5-10 min, followed by culture on ¼ MS medium with no sugar plus either 100 mg/L benlate, 0.22% Plant Preservative Mixture (PPM™; Plant Cell Technology Inc.), or no antimicrobial agent.

Sporophyte Cryopreservation

For cryopreservation, sporophytes were cut into small pieces, approximately 2-3 mm long, and transferred to ½ MS medium with and without 10 µM abscisic acid (ABA) for a 2-3 day pre-culture. In some cases, tissues were used without a pre-culture. Tissues

were then cryopreserved using two methods. For open drying, tissues were dried aseptically in glass petri plates (15 x 90 mm) on two layers of Whatman No. 1 filter paper for 4 hrs under the air flow of the laminar flow hood (average flow rate 94 fpm). For the encapsulation dehydration method (Fabre and Dereuddre, 1995), tissues were encapsulated in a 3% solution of alginic acid (PhytoTechnology Laboratories, No. A108), with a 30 minute incubation in the $CaCl_2$ solution. They were then transferred to liquid MS medium with 0.75 M sucrose and incubated overnight on a rotary shaker at 100 rpm. After 18-20 hours, the beads were dried for 4 hrs under the air flow of a laminar flow hood as for the open dried tissues, bringing moisture levels in the beads to 19-29%, depending on the experiment. After drying, some beads or open dried tissues were moved to recovery medium, as dried controls. Some beads were also used at this stage for moisture determination. Remaining beads or tissues were transferred to 2 ml polypropylene cryovials (Corning) and plunged into liquid nitrogen. Samples for testing were removed after 30-60 minutes and thawed at ambient temperature (21-23^{o}C) for 15-20 minutes, before transfer to recovery medium. Additional beads were left in LN for long-term storage in CREW's CryoBioBank. For recovery, ½ MS medium was used, in 60 x 15 mm disposable petri plates, and the tissues were incubated under the same conditions as propagating cultures. Several replicate experiments were done at different times, using tissues of different ages (time since last subculture). In one experiment, cultures of several ages were simultaneously put through the encapsulation-dehydration procedure, with and without pre-culture on ½ MS $\pm$ ABA, with recovery of dried controls and LN exposed tissues. Recovery was measured as tissue pieces with green growth after one month on recovery medium. Data were analyzed using StatView 5.0.1.

Moisture Determination

Samples were weighed (wet weight, WW), placed into an oven at 90^{o}C overnight and reweighed (dry weight, DW). The percent moisture was calculated on a wet weight basis: (WW – DW)/WW x 100.

Analysis of Total Soluble Carbohydrates

Three samples, of sporophyte tissues (stems and leaves combined) ranging from 0.03-0.17 g, were taken from cultures 2.5 months and 13.5 months in age, 3 samples of each age from 3 separate culture tubes, for analysis of total soluble carborhydrates. Tissues were individually extracted with 4 ml of boiling 80% ethanol by grinding with a Fisher PowerGen 125 at maximum speed, followed by centrifuging at 6030 x g for 10 min. The pellets were re-extracted with 4 ml of boiling 80% ethanol by vortexing with a Fisher Vortex Genie 2 at maximum setting for 15 sec and re-centrifuging. The supernatants were combined and 16 ml of chloroform and 4 ml of 0.58% NaCl were added, followed by centrifugation at 1500 x g for 10 min, to remove chlorophyll and other pigments. The aqueous phase was removed and any remaining ethanol evaporated by heating the samples on a Equatherm Temp-Blok at 90^{o}C for approximately 20 minutes. The volume of the aqueous extract was recorded, samples were diluted 1:20 and were analyzed using the anthrone method (Loewus, 1952). Absorbance at 620 nm was measured and samples were compared with a glucose standard curve.

RESULTS

Sporophyte Propagation

Sporophytes grew and propagated in the soil boxes, although growth was slow and the

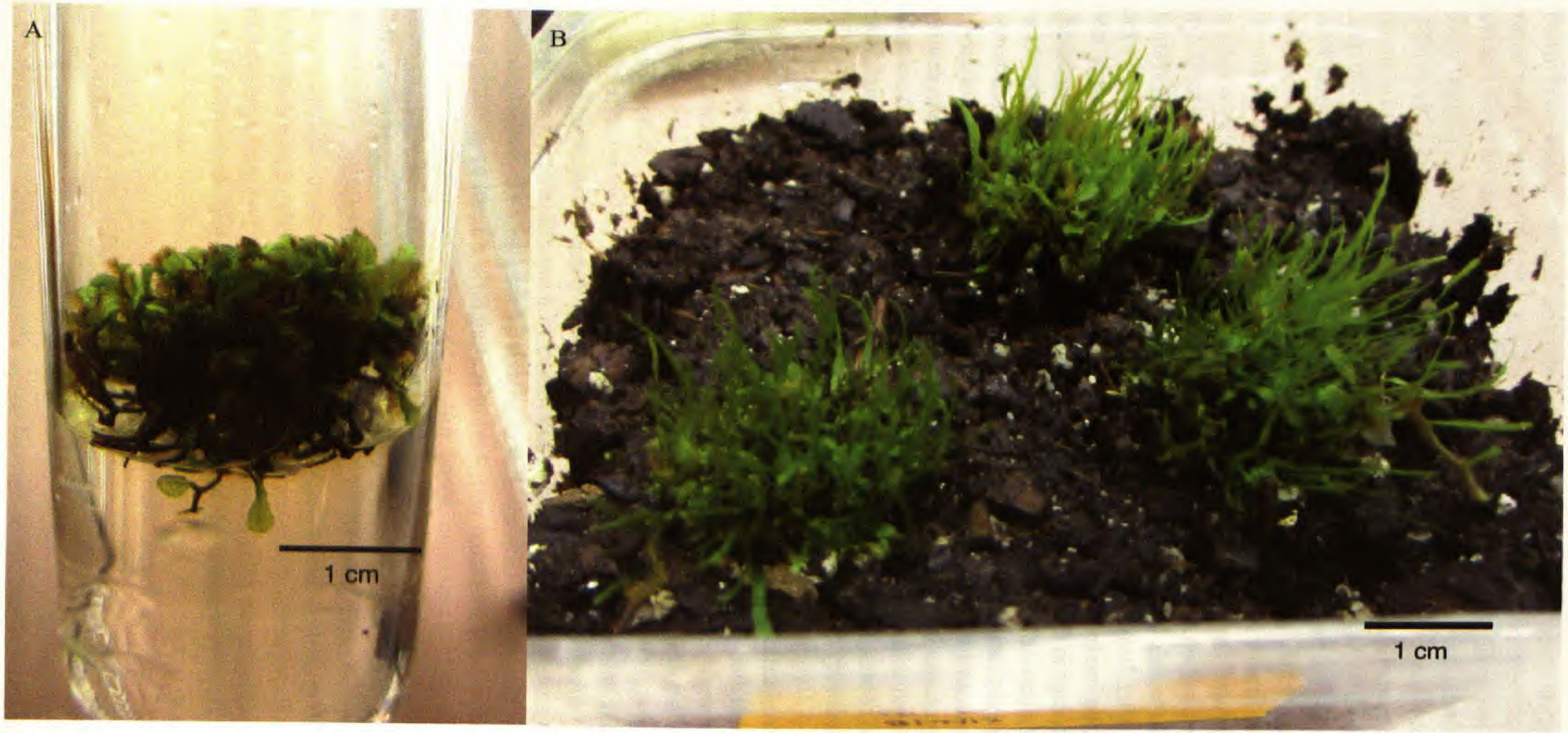

Figure 1. A. Sporophytes of *T. puncatatum* subsp. *floridanum* propagated in vitro; B. In vitro propagated sporophytes acclimatized to soil.

Table 1. Results of sterilization of *T. punctatum* sporophyte tissues for 5 minutes followed by a water rinse and culture on medium.

Sterilant	Medium	Number of Pieces	Number Clean-Appearing	No. showing growth
Bleach 1:20 dil	½ MS + Ben A	4	0	0
Bleach 1:20 dil	½ MS + Ben A	5	5	4
Bleach 1:20 dil	½ MS + Ben A	3	3	3
1% DCIC	¼ MS, 0%, Ben A	1	1	1

leaves were more elongated than in the wild. Some samples had an accompanying fungus that eventually formed a mat over the surface of the soil. This appeared to be the same fungus whenever it occurred, but it did not appear to hinder the growth of the sporophytes. The sporophytes could be separated from the fungus, rinsed, and put onto fresh soil, where they continued to grow.

Several attempts were made to surface sterilize sporophyte tissue taken from soil boxes using solutions of bleach and also of DCIC (Table 1). Although one trial with bleach was unsuccessful, both methods produced clean-appearing tissues, and a high percentage of those tissues also showed the initiation of growth. In some cases, a low level of bacteria appeared in cultures after several months, but one line that remained clean-appearing was used to establish a stock that was maintained on ½ MS medium with no benlate or antibiotics (Figure 1A), which was used to propagate plants for acclimatization. Survival of acclimatized plants was greater than 90% in the covered soil boxes (Figure 1B), although the plants did not tolerate exposure to ambient humidity levels in the laboratory.

Gametophyte Propagation

Of 12 sori cultured on agar-Ben medium, germination occurred from eight and produced green, filamentous gametophytes. These were maintained by subculture every 4-5 months onto fresh agar-Ben plates. All appeared to have some associated fungal hyphae which did not overgrow the gametophytes. After 4 months, three gametophytes had produced one sporophyte leaf each, and such leaf production continued to be observed occasionally. However, these all eventually died back and did not form propagating sporophyte cultures. In one case, one sporophyte was formed with 4 leaves. This was removed and placed into a covered soil box, but it did not survive.

Attempts to initiate sterile cultures of the gametophytes were made using surface sterilization with DCIC and recovery on medium with either no antimicrobial agents or with Ben or PPM. All tissues sterilized with 1% DCIC were totally bleached by 5 minutes, but tissues remained green through the sterilization at 0.1% and 0.01% DCIC.

After one month in culture, tissues from both 0.1% and 1% DCIC were bleached, although the tissues remained uncontaminated on control, Ben, or PPM media. Tissues sterilized with 0.01% DCIC were not bleached, but at 1 month had browned, and fungus was evident on all three recovery media.

Cryopreservation of Sporophyte Tissue

Sporophyte tissue of *T. punctatum* showed good survival through both drying and LN exposure using the encapsulation dehydration procedure and poor survival using the open drying method (Table 2; Figure 2). For each method, there was an exception, suggesting that factors other than pre-culture are also involved in survival. These exceptions did not appear to be correlated with the ages of the tissues used in this group of experiments.

In another experiment, recovery through drying and freezing was compared between tissues that were pre-cultured (with and without ABA) and those that experienced no pre-culture, among tissues of four ages, ranging from 4 to 24 months in culture. As tissues aged, survival was variable, but significantly lower at 24 months than at 4 months (Figure 3). However, even tissues that had been in culture 24 months showed a survival of about 30%. There was no significant difference at any age between survival of the dried and LN groups. There also was no difference at any age in survival of tissues pre-cultured with or without ABA in the pre-culture medium (data not shown). However, when survival was compared between tissues that received pre-culture, either with or without ABA, and those that did not have pre-culture for the four age groups, there was a significant difference between survival of pre-cultured and non-pre-cultured tissues at 4

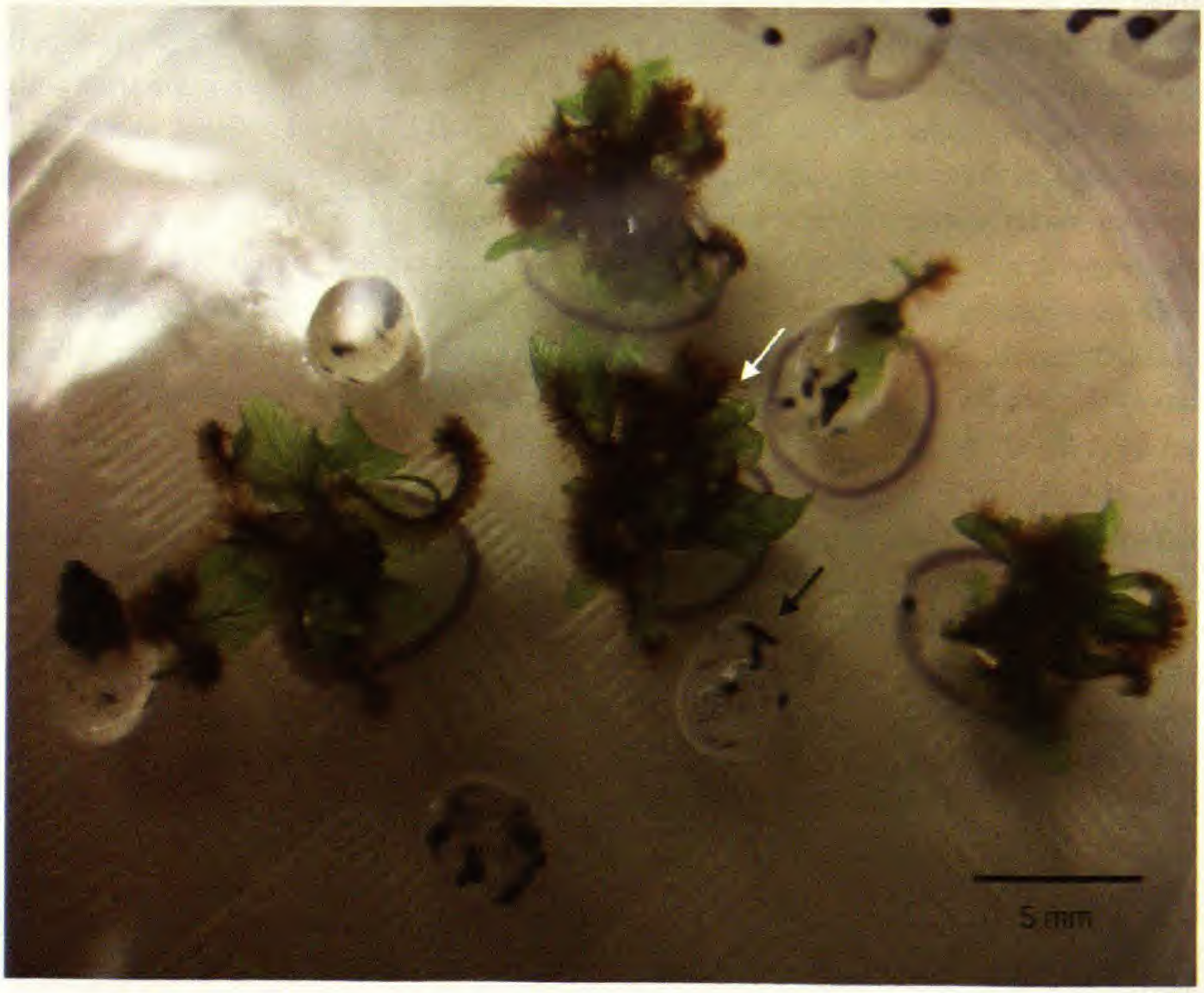

Figure 2. Sporophyte tissues on recovery medium after exposure to LN, with some pieces growing (white arrow) and others with no growth (black arrow).

Table 2. Survival of sporophyte tissues of *T. punctatum* subsp. *floridanum* through drying and LN exposure, using open drying and the encapsulation dehydration procedure. Age = months since last subculture.

Procedure	Age (mos)	Preculture with ABA	No. Dried	% Surviving Drying	No. with LN Exposure	% Surviving LN
Open Drying	3.75	-	59	8	26	0
	3.75	+	21	0	27	0
	3.0	-	4	25	6	0
	3.0	+	3	33	4	100
Encap Deh	8.5	+	11	73	10	50
	2.25	+	19	84	20	0
	3.75	-	13	84	34	68
	3.75	+	18	67	63	54
	3.0	+	10	80	11	36

and 9 months of age (Figure 4). This difference was significant only with the LN treated cultures (data not shown). There were no significant differences in survival between pre-cultured and non-pre-cultured tissues with the two older age groups.

Moisture Determination and Total Soluble Carbohydrates

Moisture levels in tissues decreased with age (Table 3). It was also observed that the medium in the older cultures had contracted, suggesting drying. Total soluble carbohydrates were also significantly lower in cultures of 13.5 months in age, compared with cultures 2.5 months in age (Table 3).

DISCUSSION

To our knowledge, this is the first report of in vitro culture of *T. punctatum* subsp. *floridanum* and of cryopreservation of sporophytes within the Hymenophyllaceae. While there have been a number of reports on cryostorage of pteridophyte gametophytes (Barnicoat *et al.*, 2011; Mikula, *et al.*, 2011; Pence, 2000), including the filmy fern, *Hymenophyllum tunbrigense* (Wilkinson, 2002), there are fewer reports on the cryopreservation of sporophytes of pteridophytes (Pence, 2001; Pence, 2014 submitted).

In the case of *T. punctatum* sporophytes, the presence of ABA in the pre-culture medium did not appear to affect subsequent survival through drying or LN exposure. This is in contrast to results with sporophytes of *Selaginella uncinata* (Desv. ex Poir) Spring and *Asplenium scolopendrium* var. *americanum* (Fernald) Kartesz & Gandhi, both of which showed improved survival with pre-culture on ABA medium (Pence, 2001;

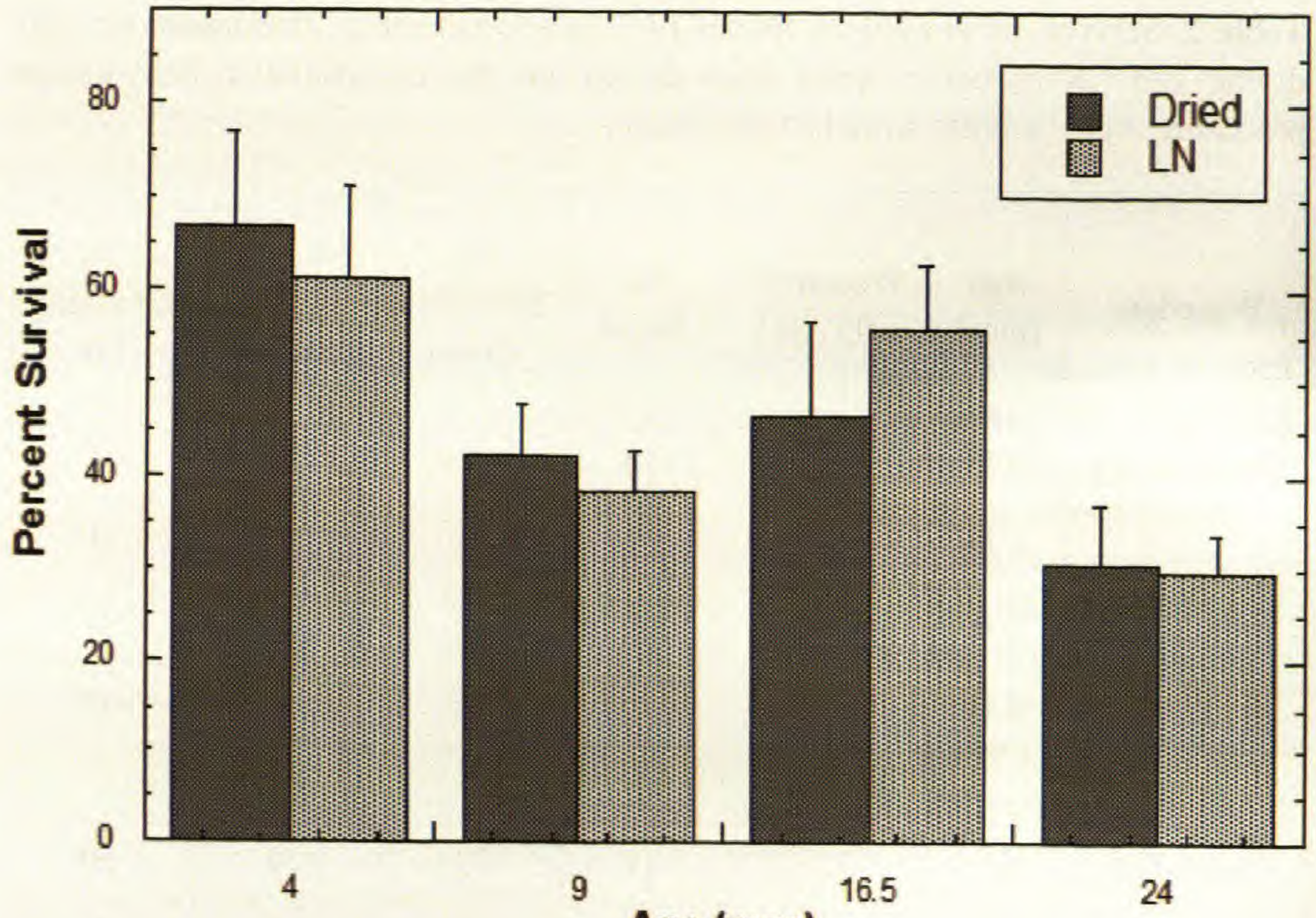

Figure 3. Percent survival of *T. punctatum* subsp. *floridanum* sporophytes of different ages through drying and LN exposure. Different letters indicate significant differences ($p < .05$).

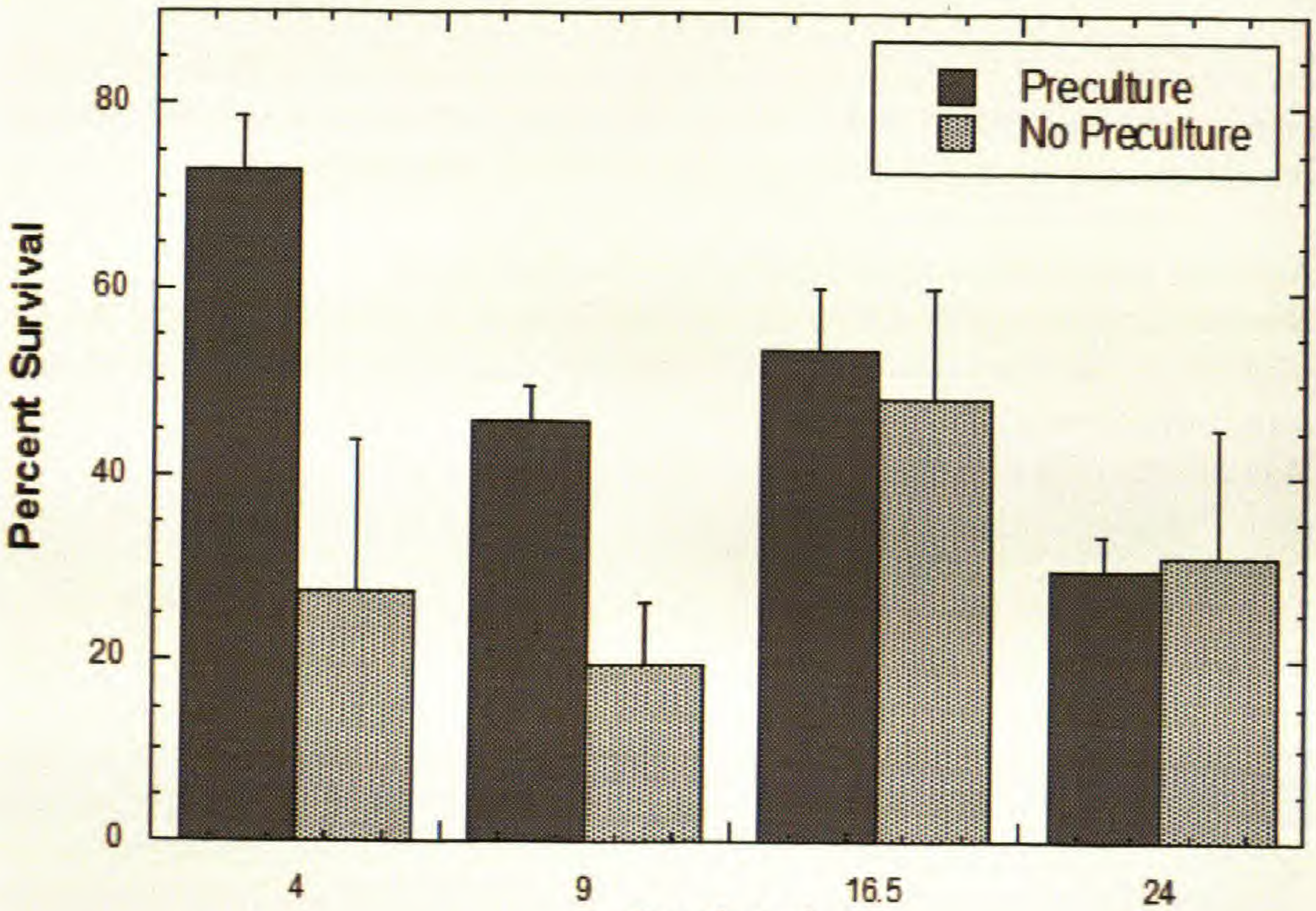

Figure 4. Percent survival through drying and LN (combined) of *T. puncatatum* subsp. *floridanum* sporophyte tissues of different ages with and without preculture. For each age, different letters indicate significant differences ($p < .05$).

Table 3. Percent moisture and total soluble carbohydrates in *T. punctatum* sporophyte tissues from 2.5 to 13.5 months in age. Within columns, different letters indicate significant differences ($p < .05$, Tukey-Kramer).

Tissue Age (mos)	Percent Moisture	Total Soluble Carbohydrates (mg/g DW glucose equivalents)
2.5	70.8 ± .011 a	223.4 ± 7.1 a
5	63.9 ± .007 b	-
8.5	66.4 ± .018 ab	-
13.5	63.8 ± .011 b	84.0 ± 38.9 b

Pence, 2014 submitted). This may relate to differences in the tissues being cryopreserved. *Trichomanes punctatum* tissues consisted primarily of sections of stem tissues with intact apical and lateral buds, from which regrowth occurred. The excised shoot tips of *S. uncinata* and bud clusters of *A. scolopendrium* require more dissection and may undergo more stress in preparation for freezing. Alternatively, there may be differences between these species in whether the tolerance mechanisms that provide for survival through the stresses of the encapsulation dehydration procedure are constitutive or inducible with ABA or other factors, as observed in relation to desiccation tolerance among species of bryophytes (Proctor & Pence, 2002).

When sporophyte cultures ranging from 4 to 24 months were examined, overall survival through desiccation and freezing was lower in the older tissues compared with younger. The moisture content of the tissues was also lower, possibly reflecting the age of the culture medium, which had contracted in volume, compared with younger cultures. Younger tissues had higher levels of total soluble carbohydrates, compared with older tissues. This could reflect depletion of nutrients in the medium with time, and could also be a factor in the greater survival of the younger tissues through drying and freezing. Total soluble carbohydrates have been correlated in several systems with an increase in tissue desiccation tolerance in both vascular plants and bryophytes (Muller *et al.*, 1997; Pence *et al.*, 2005).

Although ABA pre-culture did not appear to affect survival through cryopreservation compared with tissues pre-cultured on medium without ABA, pre-culture itself did improve survival of younger tissues compared with tissues receiving no pre-culture. Pre-culture is a common practice in cryopreservation protocols for shoot tips of angiosperms. It is often used to supply a cryoprotectant such as DMSO or a high osmoticum to the excised shoot tips (Chang & Reed, 1999), but in other cases, shoot tips are pre-cultured on a medium without cryoprotectants for several hours to a day before beginning the cryopreservation procedure (Bachiri *et al.*, 2001). Older tissues did not show this response to pre-culture, and also had a lower survival through freezing overall.

The results here suggest that producing viable sporophytic plants from gametophytes of *T. punctatum* subsp. *floridanum* may require more time and research than propagating

from sporophytes. The ability to work with the sporophyte directly provides a more rapid method for propagating and preserving this species, compared with raising plants from spores through gametophytes *ex situ*. While there are a number of fern sporophytes that have been propagated through tissue culture (Camloh & Ambrožič-Dolinšek, 2011; Pence, 2004), in vitro-grown gametophytes have been a primary focus for cryopreservation (Barnicoat *et al.*, 2011; Mikula *et al.*, 2011; Pence, 2000; Rowntree & Ramsay, 2005; Wilkinson, 2002). Our studies were not successful in establishing sterile cultures of gametophytes of *T. punctatum*, but suggest that future work with DCIC as a sterilant at concentrations between 0.01% and 0.1% might be fruitful. DCIC has been used successfully in establishing aseptic cultures of a number of bryophytes (Rowntree & Ramsay, 2005). Methods for efficiently growing healthy gametophytes that effectively produce robust sporophytes might provide more genetic diversity more readily than direct sporophyte culture.

The Species Assessment for *T. punctatum* subsp. *floridanum* cites the need for "augmentation of existing occurrences through outplantings" and "reintroduction of extirpated occurrences through outplantings" (U.S. Fish and Wildlife Service, 2012). The ability to utilize *in vitro* methods to propagate this species ex situ and to acclimatize sporophytes from *in vitro* culture to soil offers the opportunity to generate plant material for such outplantings. Work is underway on the next step: to initiate growth of the fern on a rocky substrate that can be moved to, and serve as a substrate, in the wild. In addition, as a back-up to *in situ* growth, the protocols outlined here for cryopreservation provide methods for long-term germplasm storage of the genetic diversity of this species, securing it for the future.

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HYBRIDIZATION IN *POLYSTICHUM* (DRYOPTERIDACEAE: PTERIDOPHYTA)

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Key words: synthesized hybrids, wild hybrids, meiosis, chromosome pairing, bivalents, basic chromosome number, polyploidy

ABSTRACT

The origins of two European *Polystichum* species, tetraploids *P. aculeatum* and *P. braunii*, have been investigated through experimental hybridization with other *Polystichum* species from Europe and North America, and an attempt made to re-synthesize *P. aculeatum* from its putative diploid parents, *P. lonchitis* and *P. setiferum*. The results of the hybridization programme were unexpected. Hybrids between parents which were thought to be unrelated proved easy to synthesize, and showed bivalent formation at meiosis, and chromosome pairing occurred in all hybrids examined. The degree of pairing appeared to be similar regardless of the level of ploidy. This interesting phenomenon has still not been found to occur in any other fern genus and appears to be characteristic of *Polystichum*.

INTRODUCTION

In the early nineteen-sixties a hybridization programme was set up in Leeds with the purpose of investigating the mode of origin and relationships of the two tetraploid European species of *Polystichum*, *P. aculeatum* (L.) Roth and *P. braunii* (Spenn.) Fée. In order to study the origin and relationships of a given tetraploid it is necessary first to establish whether the plant under investigation is auto- or allopolyploid, and this can most easily be done by producing wide hybrids between the tetraploid in question and species believed on morphological grounds to be unrelated to it. A longer-term aim of the project was also to synthesize *P. aculeatum* from its putative parents, the diploid species

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† Dr Anne Sleep died on 22 June 1993.

* This paper was originally prepared by Anne Sleep for the feschrift edition of the Fern Gazette (Volume X, Part X, 1987) to celebrate the 90th birthday of Professor Tadeus Reichstein, but was never completed. *Polystichum* was the genus that originally kindled Reichstein's enduring interest in the cytogenetics of ferns, and led to his long collaboration with Professor Irene Manton (e.g. Manton & Reichstein, 1961) and other colleagues from Leeds. The manuscript was still not completed at the time of Anne's untimely death in 1993, as she was still trying to discover the reason for the unusual chromosome pairing behaviour she found in *Polystichum* hybrids. However, the results themselves remain worthy of publication, especially in the light of more recent molecular studies that may shed light on the unusual chromosome pairing behaviour described here. The manuscript has been completed with some minor additions to the text and final editing by Mary Gibby (Royal Botanic Garden Edinburgh, 20A Inverleith Row, Edinburgh EH3 5LR, Scotland UK; m.gibby@rbge.ac.uk), to whom enquiries should be directed.

P. lonchitis L. and *P. setiferum* (Forssk.) Woynar. Crosses were attempted in all combinations between these four taxa, and material from elsewhere was included in the hybridization programme as and when it became available. In particular, two North American diploid species, *P. acrostichoides* (Michx.) Schott and *P. munitum* (Kaulf.) C.Presl were successfully incorporated.

The results of the hybridization programme were unexpected. Some hybrids between parents which, for both geographical and morphological reasons, were thought to be unrelated, proved easy to synthesize, and showed bivalent formation at meiosis. However, as further data accumulated, it soon became clear that some chromosome pairing occurred in all hybrids examined, and that the degree of pairing appeared to be similar regardless of the level of ploidy. This interesting phenomenon has still not been found to occur in any other fern genus and appears to be characteristic of *Polystichum*. Since the work to be described falls naturally into two parts, the original observations are first presented in the context of subsequent investigations and the significance of the chromosome pairing patterns found are discussed in relation to recent research in this and other genera. The second part of the original project, namely the attempted synthesis of *P. aculeatum*, follows the account of chromosome pairing behaviour.

MATERIAL AND METHODS

The provenance of all material used in the hybridization programme is listed in the Appendix. Voucher specimens are deposited at the Natural History Museum, London (BM). Spores were sown, prothallial cultures raised and crosses made according to the general technique developed at Leeds and described in detail by Lovis (1968a). Horticultural methods of especial relevance to *Polystichum* are described by Sleep (1966). Although putative hybrids could be recognised at an early stage by the intermediacy of their morphology, it was often four to five years, or longer, before hybrid plants became fertile and sporangia could be taken for cytological investigation. Young sporangia were fixed in a 1:3 solution of glacial acetic acid: absolute alcohol and were stored in a freezer at a temperature of -15°C until examination. Cytological squash preparations of meiosis, stained in acetocarmine, were made according to the technique of Manton (1950). Preparations were made permanent after the method of McClintock (1929). Drawings of suitable cells were made immediately, and were supplemented by photographs taken on a Reichert Biozet microscope using bright-field illumination. The photographic techniques employed in the production of chromosome diagrams and frond silhouettes are described in detail by Manton (1950).

RESULTS

The hybridization programme

The complete data relating to the hybridization programme are assembled in Table 1. Wherever possible crosses were attempted in both directions, in all cases the female parent is listed first. Prothallial cultures originating from as many different spore sources as possible were used in the attempted crosses (see column 2). Details of the localities of origin of all material used are listed in the Appendix.

Of the fifteen combinations theoretically obtainable from the basic hybridization programme incorporating the six species described above, eleven were successfully synthesized. Two crosses, namely *P. braunii* × *P. lonchitis* and *P. braunii* × *P. setiferum*, which, despite repeated attempts (756 and 592 inseminations respectively) failed to produce hybrids, are fortunately represented by naturally occurring counterparts, the wild

hybrids *P.* × *meyeri* Sleep & Reichst. (Sleep & Reichstein, 1967) and *P.* × *wirtgenii* Hahne (Manton & Reichstein, 1961). The other two hybrids not synthesized in the course of this programme both involve *P. munitum*. In view of the general ease with which both North American diploids (*P. munitum* and *P. acrostichoides*) were incorporated into synthetic hybrids (as well as the occurrence in the USA of wild hybrids involving *P. munitum* (W.H. Wagner, 1963; 1973; D. Wagner, 1979) it is rather surprising that the two combinations *P. lonchitis* × *P. munitum* and *P. setiferum* × *P. munitum* could not be obtained. Comparable numbers of inseminations were made as in the diploid – diploid cross *P. setiferum* × *P. acrostichoides*, which yielded nine hybrids from 75 inseminations.

Some crosses appear to work better in one direction than in another, although it should be borne in mind that, in view of the technical difficulty in arranging to have appropriate material available at the right stage for crossing at a particular time, in some cases many more prothalli were inseminated using one particular parent as the female. There is some indication that the tetraploids seem to cross more easily when used as the female parent, and it may be easier for the smaller spermatozoid of a diploid species to enter an archegonium of a tetraploid species.

The cytological results

The preliminary results listed in Table 1 reveal two different patterns of chromosome pairing behaviour. Two triploid hybrid combinations showed a number of bivalents that approximated closely to the base number of 41; although a preponderance of univalents was recorded in all the other hybrids, there was not one single example completely without bivalents. The literature was searched in order to discover whether further examples of this unexpected behaviour could be found. Species of *Polystichum* hybridize easily, in nature as well as in the laboratory; wild hybrids between the four European native *Polystichum* species are known and have been examined cytologically (Manton & Reichstein, 1961; Sleep, 1966; Vida, 1966; Sleep & Reichstein, 1967). Wild hybrids from North America (W. H. Wagner, 1963; 1973; D. Wagner, 1979) and Japan (Daigobo, 1974) have similarly been investigated. The North American examples show precisely the same two pairing patterns that were demonstrated by the hybridization programme; the Japanese hybrids, on the other hand, show a totally different pattern of behaviour, which will be presented separately (Table 3). Detailed cytological analyses from the synthetic hybrids listed in Table 1 are set out in Table 2, with data recorded from wild hybrids from both Europe and North America also included. Where known, the full range of pairing is given (e.g. 9-18 bivalents per cell). The figure underlined is the mean of a varying number of analyses. Where a single figure is given it usually represents a single analysis (obtained, perhaps, before a delicate plant died), although it can also represent a literature citation of one particular analysis given where other cells were observed to give pairing of a similar order. The suggested genome representation (column 2) is in most cases based on the initial letter of the species involved; in the case of the North American Polystichums the scheme of Wagner (1973) is followed for the sake of consistency and ease of cross-reference to his work.

There is a further source of information concerning chromosome pairing behaviour in *Polystichum*, and this is the cytological investigation by Daigobo (1974) of a series of triploid and tetraploid wild hybrids from Japan. In Japan all the large bipinnate species of *Polystichum* (i.e. those resembling *P. setiferum* and numbering no less than 14 distinct species) are classified together in the section Metapolystichum Tagawa (Daigobo, 1972). There occur also seven simply pinnate taxa (Tagawa, 1940; 1959; Daigobo, 1972;

Table 1: Result of Hybridization Programme

Cross	No. of different parental cultures	Prothalli inseminated	Sporelings obtained	Selfs	Died	Hybrids Obtained	Percentage success	Cytology (preliminary analysis)
DIPLOID HYBRIDS								
♀ *P. setiferum* (2x) × *P. lonchitis* (2x)	3 3	95	3	3	0	0	-	-
♀ *P. lonchitis* (2x) × *P. setiferum* (2x)	8 4	878	112	17	55	40	4.55 %	Many univalents (c.50) few abnormal bivalents
♀ *P. lonchitis* (2x) × *P. acrostichoides* (2x)	5 2	175	7	5	1	1	0.57 %	c. 25 irregular paired chromosomes
♀ *P. lonchitis* (2x) × *P. munitum* (2x)	2 1	75	2	2	0	0	-	-
♀ *P. munitum* (2x) × *P. lonchitis* (2x)	1 1	16	1	1	0	0	-	-
♀ *P. setiferum* (2x) × *P. acrostichoides* (2x)	3 2	75	14	3	2	9	12.0 %	Many univalent plus a few abnormal pairs
♀ *P.acrostichoides* (2x) × *P. setiferum* (2x)	1 1	10	0	0	0	0	-	-
♀ *P. setiferum* (2x) × *P. munitum* (2x)	2 1	55	4	4	0	0	-	-
♀ *P. munitum* (2x) × *P. setiferum* (2x)	1 2	24	0	0	0	0	-	-
♀ *P.acrostichoides* (2x) × *P. munitum* (2x)	2 2	106	7	3	1	3	2.83 %	16 bivalents and many univalents
♀ *P. munitum* (2x) × *P. acrostichoides* (2x)	2 2	120	3	2	1	0	-	-
TRIPLOID HYBRIDS								
♀ *P. aculeatum* (4x) × *P. lonchitis* (2x)	4 5	126	17	13	0	4	3.17 %	40-41 paired chromosomes and c.41 univalents
♀ *P. lonchitis* (2x) × *P. aculeatum* (4x)	2 1	55	10	1	2	7	12.73%	

Table 1: continued

♀ *P. aculeatum* (4x) × *P. setiferum* (2x)	24	135	52	0	0	52	38.52 %	39-41 paired chromosomes and 41-45 univalents
♀ *P. setiferum* (2x) × *P. aculeatum* (4x)	23	58	0	0	0	0	-	-
♀ *P. aculeatum* (4x) × *P. acrostichoides* (2x)	32	63	30	4	4	22	34.92 %	Many univalents (c.100) few abnormal pairs
♀ *P. aculeatum* (4x) × *P. munitum* (2x)	31	148	54	7	5	42	28.38 %	Many univalents; a small number of bivalents also present
♀ *P. braunii* (4x) × *P. lonchitis* (2x)	68	561	38	22	16	0	-	-
♀ *P. lonchitis* (2x) × *P. braunii* (4x)	44	195	19	16	3	0	-	-
♀ *P. braunii* (4x) × *P. setiferum* (2x)	85	490	29	16	13	0	-	-
♀ *P. setiferum* (2x) × *P. braunii* (4x)	24	102	1	1	0	0	-	-
♀ *P. braunii* (4x) × *P. acrostichoides* (2x)	71	224	21	7	8	6	2.68 %	Some hybrids proved to be 4x not 3x as expected
♀ *P. acrostichoides* (2x) × *P. braunii* (4x)	11	6	0	0	0	0 -		-
♀ *P. braunii* (4x) × *P. munitum* (2x)	52	244	35	13	9	13	5.33 %	Many univalents and some irregular pairs.
♀ *P. munitum* (2x) × *P. braunii* (4x)	11	16	0	0	0	0 -		-
TETRAPLOID HYBRID								
♀ *P. braunii* (4x) × *P. aculeatum* (4x)	32	58	6	3	2	1	1.72 %	Many univalents; c.10-20 abnormal pairs.
♀ *P. aculeatum* (4x) × *P. braunii* (4x)	23	88	6	6	0	0 -		-

Table 2. Bivalent formation in *Polystichum* hybrids from Europe and North America

Cross	Suggested genome representation	Synthetic or wild	Range of pairing and mean (underlined)	Reported by
Diploid hybrids:				
P. lonchitis (2x) × *P. setiferum* (2x)	SL	Synthetic	6 – <u>15</u> - 28	Sleep, 1966
P . ×lonchitiforme Halacsy = *P. lonchitis* × *P. setiferum* (2x)	SL	Wild (Ireland)	c. 18	Sleep, unpublished
P . × lonchitiforme Halacsy = *P. lonchitis* × *P. setiferum* (2x)	SL	Wild (Hungary)	c. 21	Vida & Pinter, 1981
P. lonchitis (2x) × *P. acrostichoides* (2x)	LA	Synthetic	25	Sleep, 1966 pp. 182, 189, 241
P. setiferum (2x) × *P. acrostichoides* (2x)	SA	Synthetic	9 – <u>13</u> – 18	Sleep, 1966 pp. 183,188, 242
P. acrostichoides (2x) × *P. munitum* (2x)	AR	Synthetic	16	Sleep, 1966
P. munitum (2x) × *P. imbricans* (2x)	RI	Wild (USA)	3-11	D.H. Wagner, 1979
P. dudleyi (2x) × *P. munitum* (2x)	DR	Wild (USA)	19 – <u>26</u> – 33	W.H. Wagner, 1973
P. mohrioides (2x) × *P. munitum* (2x)	MR	Wild (USA)	2 – <u>24</u> – 30	W.H. Wagner, 1973
P. speciosissimum (A.Braun ex Kunze) Copel. (2x) × *P. muricatum* (L.) Fée (2x)		Wild (Costa Rica)	10	Barrington, 1985

Table 2. continued

Triploid hybrids:				
P. aculeatum (4x) × *P. munitum* (2x)	SLR	Synthetic	19 – <u>20</u> – 22	Sleep, 1966 pp. 184, 193, 244
P. aculeatum (4x) × *P. acrostichoides* (2x)	SLA	Synthetic	9 – <u>14</u> – 18	Sleep, 1966 pp. 192, 244
P. × *meyeri* Sleep & Reichst. = *P. lonchitis* (2x) × *P. braunii* (4x)	LXY	Wild	6 - <u>15</u> – 24	Sleep & Reichstein 1967
P. × *wirtgenii* Hahne = *P. setiferum* (2x) × *P. braunii* (4x)	SXY	Wild	12	Manton & Reichstein, 1961
P. × *wirtgenii* Hahne = *P. setiferum* (2x) × *P. braunii* (4x)	SXY	Wild	12 – <u>14</u> – 17	Sleep, 1966 p. 196
P. braunii (4x) × *P. acrostichoides* (2x)	XYA	Synthetic		Sleep, 1966
P. × *potteri* Barrington = *P. braunii* (4x) × *P. acrostichoides* (2x)	XYA	Wild	18 – <u>22</u> – 26	Barrington, 1986
P. × *hokurikuense* Sa.Kurata = *P. longifrons* (4x)× *P. retrosopaleaceum* (2x)		Wild	21 - 22	Daigobo, 1974
Tetraploid hybrids:				
P. × *luerssenii* (Dörfl.) Hahne = *P. aculeatum* (4x) × *P. braunii* (4x)	SLXY	Wild	9	Manton & Reichstein, 1961
P. ×*luerssenii* (Dörfl.) Hahne = *P. aculeatum* (4x) × *P. braunii* (4x)	SLXY	Wild	10 – <u>13</u> - 18	Sleep, 1966 p. 194
P. aculeatum (4x) × *P. braunii* (4x)	SLXY	Synthetic	10 - 20	Sleep, 1966 pp. 186, 245
Pentaploid hybrid:				
P. setiferum (2x) × *P. falcinellum* (8x)		Wild	18 - 32	Lovis in Manton *et al.* 1986

Table 3. Chromosome pairing in Japanese wild hybrids (adapted from Daigobo, 1974)

Triploid hybrids	Range of bivalent formation
P. × *amboversum* Sa.Kurata = *P. retrosopaleaceum* (Kodama) Tagawa (2x) × *P. ovatopaleaceum* (Kodama) Sa.Kurata (4x)	40 – 41
P. × *hitoyoshienese* Sa.Kurata = *P. otomasui* Sa.Kurata (2x) × *P. pseudomakinoi* Tagawa (4x)	40 – 41
P. × *hokurikuense* Sa.Kurata = *P. retrosopaleaceum* (2x) × *P. longifrons* Sa.Kurata (4x)	21 – 22
P. × *inadae* Sa.Kurata = *P. retrosopaleaceum* (2x) × *P. polyblepharum* (Roem. ex Kunze) C.Presl (4x)	45
P. × *jitaroi* Sa.Kurata = *P. fibrillosopaleaceum* (Kodama) Tagawa (2x) × *P. pseudomakinoi* (4x)	30 – 31
P. × *kumamontanum* Sa.Kurata = *P. otomasui* (2x) × *P. polyblepharum* (4x)	38 – 39
P. × *miuranum* Sa.Kurata = *P. fibrillosopaleaceum* (2x) × *P. polyblepharum* (4x)	40 – 41
P. × *ohtanii* Sa.Kurata = *P. fibrillosopaleaceum* (2x) × *P. longifrons* (4x)	35 – 38
P. × *shintashiroi* Sa.Kurata = *P. retrosopaleaceum* (2x) × *P. microchlamys* (Christ) Matsumura (4x)	32
P. × *suginoi* Sa.Kurata = *P. otomasui* (2x) × *P. tagawanum* Sa.Kurata (4x)	32 – 42
P. × *utsumii* (Sa.Kurata) Sa.Kurata = *P. retrosopaleaceum* (2x) × *P. pseudomakinoi* (4x)	24 – 32

Nakaike, 1975), six of which are known to be diploid (Kurita, 1967; Mitui, 1966; 1967b; Diagobo, 1973); these are rare species of rocky habitats and they do not appear to cross, either with each other or with any of the bipinnate *Polystichum* species. Of the bipinnate species belonging to the section Metapolystichum only five are diploid (Kurita, 1966a; Mitui, 1965; 1968; Sleep, 1966; Diagobo, 1973); these appear to be isolated from each other both ecologically and geographically as hybrids between them are unknown.

Table 3. continued

Tetraploid hybrids	Range of bivalent formation
P. × *hakonense* Sa.Kurata = *P. longifrons* (4x) × *P. pseudomakinoi* (4x)	52
P. × *izuense* Sa.Kurata = *P. makinoi* (4x) × *P. tagawanum* (4x)	63
P. × *kiyozumianum* Sa.Kurata = *P. pseudomakinoi* (4x) × *P. tagawanum* (4x)	56
P. × *kunioi* Sa.Kurata = *P. braunii* (4x) × *P. makinoi* (4x)	47
P. × *kurokawae* Sa.Kurata = *P. makinoi* (4x) × *P. ovatopaleaceum* (4x)	50 – 59
P. × *mashikoi* Sa.Kurata = *P. polyblepharum* (4x) × *P. tagawanum* (4x)	51 – 52
P. × *ongataense* Sa.Kurata = *P. ovatopaleaceum* (4x) × *P. pseudomakinoi* (4x)	50
P. × *namegatae* Sa.Kurata = *P. makinoi* (4x) × *P. pseudomakinoi* (4x)	50 - 55

Although no diploid hybrids have been recorded from Japan[1], hybridization does nevertheless occur between at least three of these bipinnate diploids and the tetraploid bipinnate species assigned to Metapolystichum. Indeed, within this group hybridization is rife, with no less than 25 hybrids having been described by Kurata (1964). Of these, 19 have been examined cytologically by Daigobo; the bivalent formation recorded by him in 11 triploid and eight tetraploid hybrids is presented in Table 3.

Chromosome pairing behaviour: interpretation and discussion

An examination of the results set out in Table 2 clearly reinforce the two distinct patterns of chromosome pairing behaviour already discernible from the preliminary analyses from synthetic hybrids that were listed in Table 1. Six hybrid combinations in which the bivalent formation approximates to 41 exhibit the well-known and familiar pattern of 'n' paired and 'n' single chromosomes ('n' being the base number of the genus in

[1] Recently the difficulty in recognising diploid *Polystichum* hybrids from Japan has been described by Lin *et al.* (2011)

question) that has been recorded in triploid hybrids in many different fern genera. Although it should always be borne in mind that a triploid hybrid between an autotetraploid and any diploid species unrelated to it can also show 'n' paired and 'n' single chromosomes (Lovis, 1977; Sleep, 1980; 1983), this pairing pattern, particularly when supported by morphological evidence, provides a very reliable indication of a part-parental relationship between an allotetraploid species and one of its diploid progenitors. That the two triploid hybrids, *P. aculeatum* × *P. lonchitis* and *P. aculeatum* × *P. setiferum*, should regularly show c.41 paired and 41 single chromosomes at meiosis in both wild and synthetic examples is not therefore unexpected in view of the morphological intermediacy of *P. aculeatum* between *P. lonchitis* and *P. setiferum*, and the hypothesis of Manton (1950) that each of these two diploids is part-parental to *P. aculeatum*. No less than four North American triploid hybrids *P. californicum* (D.C.Eaton) Diels × *P. dudleyi* Maxon, *P. californicum* × *P. munitum*, *P scopulinum* (D.C.Eaton) Maxon × *P. mohrioides* (Bory ex Willd.) C.Presl and *P. scopulinum* × *P. munitum*, also show the pattern of 'n' paired and 'n' unpaired chromosomes already observed in the back-cross hybrids between *P. aculeatum* and its putative ancestors. The results from these North American wild hybrids have already been interpreted (Wagner, 1963; 1973) as indicating a part-parental relationship between the two tetraploids in question and their respective diploid progenitors. There can be no doubt that this interpretation is correct in view, in each case, of the existence in the field, alongside the tetraploid and the back-cross hybrids, of the sterile diploid hybrid which is morphogically indistinguishable from the tetraploid.

It is also interesting to note that both the North American wild diploid hybrids, *P. dudleyi* × *P. munitum* (sterile *P. californicum* of Wagner) and *P. mohrioides* × *P. munitum* (sterile *P. scopulinum* of Wagner) show some degree of bivalent formation and exactly parallel the behaviour observed in the diploid hybrids (wild and synthetic) between *P. lonchitis* and *P. setiferum*, although in both instances the North American diploid hybrids show a slightly higher range of bivalent formation than does *P. lonchitis* × *P. setiferum*.

That any chromosome pairing at all occurs in these two crosses and in the remainder of the wide hybrid combinations listed in Table 2 is surprising, especially in view of the gross morphological differences between *P. setiferum* and the other diploid species, and the fact that the two simply pinnate North American diploids would not be expected to have any close affinity with either of the European tetraploids, *P. aculeatum* and *P. braunii*. Nevertheless, the most striking feature of the results assembled in Table 2 is that in none of the wide hybrid combinations listed is there a single instance of the complete failure of chromosome pairing which has frequently been observed in hybrids between unrelated species in other fern genera. In the diploid hybrids, out of a possible maximum of 41, the range of bivalent formation is from 6 (exceptionally 3, in one case) to 33, with a mean value falling between 15 and 25. Surprisingly, the range of pairing is seemingly independent of the level of ploidy, being of a similar order in diploid, triploid and tetraploid hybrids.

Firstly we may ask if this chromosome pairing, observed so widely in hybrids between presumably unrelated species, is true bivalent formation or some sort of false association as a result of some physiological or chemical disturbance. A mutant of *Ceratopteris* with 'sticky' chromosomes has been described (Hickock, 1977), but his report deals with a single apogamously derived sporophyte from a triploid hybrid and does not appear to be relevant to the situation observed in *Polystichum*. The latter genus

does certainly have some tendency towards 'stickiness' in its chromosomes and it is quite difficult to obtain cells from *Polystichum* in which the chromosomes are clear, well squashed and well spread. Although some of the wide hybrids show very loosely associated pairs and, in metaphase figures, straggling and uncoiling bivalents, others show the typical cross- and ring-shaped bivalents having either one or two chiasmata and these are indistinguishable from similar associations seen in the regular meioses of all *Polystichum* species (see, for example, figures in Wagner (1973), Vida (1966), and Barrington (1986). It is thus concluded that the associations observed do represent true chromosome pairings with both chiasmata formation, and presumably recombination, occurring.

A small amount of pairing between supposedly unrelated genomes might be explained in terms of structural alterations or interchanges of chromosomal segments over a period of time, but the phenomenon appears to be of such general occurrence throughout the genus that this possibility is extremely unlikely. Furthermore, the fact that homologous chromosomes pair completely in the several examples of back-cross hybrids listed in Table II shows that in the three tetraploids discussed there has been little differentiation or structural change between their genomes and those of their respective parental diploids. With the exception of the back-cross hybrids showing c.41 bivalents, the chromosome pairing which has been observed in all the rest of the hybrids listed in Table 2 is more satisfactorily interpreted as pairing between homoeologous (meaning similar, but not identical) chromosomes (Huskins, 1931). Presumably all the present-day diploids have evolved from a common ancestor by a process of evolutionary divergence. In *Polystichum* such evolution appears to have produced a large number of diploid species of distinctive morphology which are isolated from one another both geographically and ecologically, but not cytologically. That there should still be some residual homology between the chromosomes of such strikingly morphologically different diploid species as *P. lonchitis* and *P. setiferum*, or *P. dudleyi* and *P. munitum*, is surprising. That cytological and morphological differentiation need not necessarily proceed at the same rate has, however, been pointed out by Stebbins (1972), and certainly in *Polystichum* differentiation at the cytological level does not seem to have kept pace with morphological divergence, since the diploid species so far tested all seem to have retained some degree of cytological homology and are thus able to produce bivalents when they are brought together in hybrids.

When the observations assembled in Table 2 are examined in closer detail, we see that the results from the diploid hybrids show that the genome A (in *P. acrostichoides*) has a partial homology, not only with R, but with L and S also. We know too that S has some homology with L as well as with A. Similar homologies can be inferred from the chromosomes pairing observed in the triploid hybrids. Of these, *P. aculeatum* × *P. acrostichoides* is the most interesting example. Its genomic constitution may be represented by the letters SLA. From the evidence supplied by the synthetic diploid hybrids we know that all three of its constituent genomes show residual homologies with each of the other two, and are capable of pairing, at least in part, with one another. One might therefore expect to see some trivalents formed between the homoeologous chromosomes of the S, L and A genomes. In fact, the same range of bivalent formation as in the diploid hybrids is observed, and there is no sign of multivalent associations. This result can perhaps best be explained on the basis of preferential pairing and the fact that bivalents are a more stable configuration than trivalents. Once bivalent associations have been formed and some sort of equilibrium reached, the remaining chromosomes,

even those having homologous segments with chromosomes already involved in bivalent association, will appear as univalents.

Consideration of the tetraploid hybrid, *P. aculeatum* × *P. braunii*, shows that in this hybrid too the range of bivalent formation is similar to that recorded from the diploid and triploid hybrids, although because of its tetraploid level a higher number (c.120-140) of univalent chromosomes is also present. From the evidence of the triploid hybrids involving *P. braunii*, two interpretations are possible, (i) that *P. braunii* is an ancient tetraploid whose constituent genomes, designated X and Y, have diverged over a very long period of time so that there now remains only the very weak homology between them. This would be sufficient to produce the very irregular bivalents observed in hybrids between *P. braunii* and other, morphologically unrelated, taxa. But, on this view, since hybrids between *P. lonchitis* and *P. setiferum* are known to be potentially capable of forming up to 28 pairs, a higher number of bivalents than the observed maximum of 20 would be expected in the hybrid *P. aculeatum* × *P. braunii*. On the other hand, (ii) the behaviour of *P. braunii* in triploid hybrids so closely resembles that already observed in the parallel series of combinations with *P. aculeatum* that it seems likely that in each case the underlying cause is the same. If this is so, it follows that in the hybrid *P. aculeatum* × *P. braunii* the same chromosomes in each of its four constituent genomes retain a homology with each other and that part of the S genome is potentially capable of pairing with its counterparts in each of the L, X and Y genomes.

In this case, multivalent associations would be expected. That they do not occur can be explained in either genetic or physical terms. It is possible that some genetic system is operating which suppresses or inhibits the formation of multivalent associations. Alternatively, it may be that the chromosomes are too small to allow the regular formation of tri- and quadrivalents. In the fern genus *Asplenium* the chromosomes are similarly small in size and, as in *Polystichum*, bivalents having one or two chiasmata are the rule. In *Asplenium*, however, multivalents are regularly formed when duplicated genomes are present, as in back-cross hybrids between autotetraploids and their putative progenitors (Lovis, Sleep & Reichstein 1969; Sleep, 1980), and it therefore seems unlikely that the lack of multivalents in *Polystichum* has a physical cause. Even if multivalent formation is suppressed genetically, a higher number of bivalents would, on the basis of the homologies which can be postulated to exist, still be expected. The reason why a higher number is not realised is not at all clear.

Similar behaviour is recorded also from a wild pentaploid hybrid, *P. setiferum* × *P. falcinellum* (Lovis in Manton *et al.*, 1986) from the island of Madeira. *Polystichum falcinellum*, a simply pinnate species superficially rather similar to *P. munitum*, and endemic to Madeira, is octoploid (Manton, 1950). The wild hybrid with *P. setiferum* shows a preponderance of univalents and 18-37 paired chromosomes, a range of bivalent formation which closely parallels results from other hybrids listed in Table 2. The bivalents are very irregular in shape and similar to those observed in hybrids involving *P. braunii*. Weak homologies may be present either within the constituent genomes of *P. falcinellum* or between one or more of the genomes of *P. falcinellum* and *P. setiferum* and in either case a higher degree of bivalent formation, or the presence of multivalent associations, might be expected. Although the potential for forming such associations may be present but suppressed through the operation of some genetic mechanism as has been postulated above with regard to the hybrid *P. aculeatum* × *P. braunii*, it is also possible that in *P. falcinellum*, undoubtedly a species of great antiquity, its constituent genomes have diverged so far that any residual homology between them has been lost.

The occurrence of such widespread chromosome pairing in a large number of hybrids between supposedly unrelated species, in particularly those at the diploid level, is a phenomenon not previously encountered in the Pteridophyta. The only other fern genus which has been investigated in comparable detail is *Asplenium*. In this genus there is in Europe at the present time a large number of diploid species well differentiated from each other both morphologically and cytologically, and when crossed together the diploid hybrids invariably show complete failure of chromosome pairing. This behaviour has been recorded in wild examples (Lovis, Melzer & Reichstein, 1965; Lovis & Reichstein, 1968a; 1968b; Vida & Reichstein, 1971; Lovis unpubl., quoted in Reichstein, 1981; Badré *et al.*, 1981) as well as in deliberately synthesized hybrids (Lovis, 1968b; Shivas, 1969; Lovis, 1970). There are also very many well documented examples of complete failure of chromosome pairing in triploid hybrids between unrelated species in this genus (see, for example, Lovis & Vida, 1969; Lovis *et al.*, 1972; Sleep, 1983). Complete failure of pairing in diploid hybrids has also been recorded from the following genera: *Adiantum* (Manton, Ghatak & Sinha, 1967), *Athyrium* (Schneller & Rasbach, 1984), *Cheilanthes* (Vida, Major & Reichstein, 1983) and *Dryopteris* (Manton & Walker, 1953; Wagner & Hagenah, 1962; Gibby & Walker, 1977; Gibby *et al.*, 1978). Similarly, triploid hybrids in these genera show two broad patterns of chromosome pairing behaviour, namely, in back-cross hybrids with a putative ancestor, the familiar pattern of 'n' paired with 'n' univalent chromosomes, and in crosses between supposedly unrelated species virtually complete failure of chromosome pairing. There has as yet been no indication in any other genus so far investigated of the widespread pairing between homoeologous chromosomes which is so characteristics of *Polystichum*.

The contrast with *Dryopteris* is particularly apposite in view of the fact that both *Polystichum* and *Dryopteris* share the same base number, namely 41, and so are presumably related. In *Dryopteris*, 82 unpaired chromosomes have been reported from at least five different diploid hybrids (see references above), although there is at least one record in the literature of some bivalent formation between particular pairs of diploid species. A good example of this behaviour is shown by the wild diploid hybrid, *D.* × *initialis* Fraser-Jenk. & Corley (Gibby in Fraser-Jenkins, 1976). Here some 5-23 pairs (mean 14) are regularly recorded, and it is probable that in this case there is incomplete differentiation between the genomes of *D. oreades* Fomin and *D. caucasica* (A.Braun) Fraser-Jenk. & Corley, the two diploid species involved. However, such behaviour seems to be limited to isolated examples, the broad trend tending to be, in the species so far investigated, towards complete cytological differentiation at the diploid level.

The present state of our knowledge thus suggests that the homoeologous pairing so widely observed in *Polystichum* hybrids may well be a unique feature amongst the ferns; further research in this and other genera is highly desirable. In the meantime, the remarkably constant degree of homoeologous pairing between genetically differentiated genomes at the diploid level in *Polystichum* has led Lovis (1977) to propose an explanation in terms of some intrinsic property of the ancestral *Polystichum* genome. He suggests that certain chromosomes or groups of chromosomes have, perhaps because the particular combination of genes which they carry has some curiously felicitous advantage to the genus as a whole, been conserved against extensive change in some way and have actually been maintained in their original state by selection so that they are capable of recognizing each other and forming bivalents when they are brought together in hybrids.

This is a plausible hypothesis, although the occurrence of a similar degree of pairing in diploid, triploid, tetraploid and pentaploid hybrids still remains for which there is, as

yet, no satisfactory explanation.

Consideration of the results from the Japanese hybrids shows an apparently very different picture. As can be seen by reference to Table 3, the chromosome pairing behaviour in the eleven triploid hybrids listed is very variable. It also very difficult to interpret, as the pairing extends from one combination showing 21-22 bivalents, through ranges of bivalent formation in the thirties, to some combinations in which the pairing approximates to the monoploid number of 41. In the latter case it is tempting to suggest that, as in the European and North American examples exhibiting the same pattern, this represents pairing between the allotetraploid species and one of its ancestors, but I believe that in these Japanese examples such an explanation is not the correct one. The section Metapolystichum comprises at least two species-complexes, one based on the diploid, *P. retrosopaleaceum* (Kodama) Tagawa, and another on *P. fibrillosopaleaceum* Tagawa. In both these groups there are tetraploids that are morphologically very similar to the diploids, and it is likely that autopolyploidy, such as has been demonstrated in some European *Asplenium* species (Lovis, 1977; Sleep, 1983), has played a part in their evolution. If this is the case, then some of the bivalent formation observed in the triploid hybrids could well be due to autosyndesis. A closer examination of the numbers of bivalents recorded from the tetraploid hybrids lends support for this view. In seven of the eight combinations examined by Daigobo (1974), the numbers of bivalents are remarkably constant, ranging from 50 to 63 per cell. The one possible exception, the hybrid *P. braunii* × *P. makinoi* (listed as *P.* × *kunioi* Sa.Kurata), shows 47 bivalents, in contrast with the 9-20 observed in the parallel European hybrid *P. aculeatum* × *P. braunii*. It is likely that the number of 47 is made up of autosyndetic pairs within the two genomes contributed by the *makinoi* parent, plus some homoeologous pairs arising from the *braunii* parent. The amazingly similar level of bivalent formation observed in the remainder of the wild tetraploid hybrids investigated by Daigobo (50-63 pairs) could be due to autosyndesis within the constituent genomes contributed by each parent, plus homoeologous pairing within the genomes of the other parent. Certainly the relationships between the Japanese species of the Section Metapolystichum are complex, and their inter-relationships can be elucidated only by a controlled programme of experimental breeding and the production of 'wide hybrids' with species which are clearly unrelated. Some evidence that duplicated genomes are in fact present is available from a close examination of the chromosome photographs in Daigobo's 1974 paper. Although he states that 'a few trivalent-like chromosomes were observed in three triploid hybrids' but that the presence of multivalents 'is not confirmed', there do seem to be associations which have the characteristic appearance of multivalents in some of the cells (both in triploid and tetraploid hybrids) illustrated, and it is very likely that some do in fact occur.

Although Daigobo (1974) offers no explanation for his results, his observations are relevant to this discussion for the two following reasons: firstly, they establish a third pattern of chromosome pairing behaviour in *Polystichum*, a pattern which has not yet been observed in any of the European or North American wild or synthetic hybrids so far investigated there (see data assembled in Table 2). Only one hybrid, namely *P.* × *hokurikuense* Sa.Kurata (= *P. longifrons* Sa.Kurata x *P. retrosopaleaceum*), shows pairing which approximates to that found by Sleep and others in hybrids between unrelated species, and which can be interpreted as pairing between homoeologues. Secondly, there appears to be a distinction between the consistent observation of c.50-60 bivalents in all the tetraploid Japanese hybrids investigated and the small number (>20) of bivalents regularly displayed in the European tetraploid hybrids between *P. aculeatum* and

P. braunii. These contrasting observations may possibly be explained in terms of the different origins of the tetraploids involved if, as suggested here, autopolypoidy has indeed played a significant role in the evolution of the Japanese tetraploids.

The attempted synthesis of *P. aculeatum*

The diploid hybrid between *P. lonchitis* and *P. setiferum* proved, despite the disparity in appearance of these two species, easy to synthesize, and 40 hybrid plants were obtained. The cross worked better using *P. lonchitis* as the female parent, and success appeared to be independent of the geographical origin of the material, parental stocks from Switzerland, Scotland, Romania and North America all being successfully combined with European *P. setiferum*. Attempts made to induce chromosome doubling in the young sporangia of these diploid hybrids by treatment with colchicines solutions of varying strengths (Sleep, 1966) were unsuccessful, but parallel experiments made by Reichstein (unpublished) to raise an F2 generation directly by sowing the spores of the diploid hybrid met, eventually, with more success. It took several years to obtain sporophytes from the sowings of the diploid hybrids, and eventually some seven plants were raised to maturity. One of these was examined cytologically by Vida (unpublished); the chromosome number was found to be 2n =164 and it produced good spores. In most cells meiosis was completely regular, with 82 bivalents; occasionally a few univalents were observed. There was no sign either of multivalents or of the irregular associations which are characteristic of the diploid hybrid. A wild diploid hybrid between *P. lonchitis* and *P. setiferum*, *P.* × *lonchitiforme* (Halàcsy) Bech., which was found in Ireland in 1974 (Sleep, 1976; Vida & Pinter, 1981), showed similar pairing behaviour (Sleep & Souter, Paloma Cubas pers. comm.) to that already recorded from the synthetic diploid hybrids made by Sleep (1966). The wild Irish examples, in contrast to the rather weak and stunted synthetic diploids, were large, robust plants, and a fine example of *P.* × *lonchitiforme* was brought back from Ireland and established in cultivation at Leeds. Spores from this diploid hybrid, although they appeared to be mainly abortive, germinated readily and gave a good crop of prothalli which, on being flooded with water to encourage self-fertilization, yielded numerous sporophytes. These, in contrast to the few sporelings obtained with so much effort from the synthetic diploid hybrids, grew strongly, and more than 30 specimens have been cultivated in the experimental garden of the Leeds University Plant Sciences Department for many years. These F2 plants will all be homozygous, and it is therefore not surprising that they are all closely similar in appearance, each individual being a virtually identical replica of the single diploid hybrid from which it arose. All of these plants have been examined cytologically (Sleep) and shown to be tetraploid. Meiosis has been examined in many cells from each individual, and observations made over a period of several years. Without exception each plant shows a regular meiosis with 82 perfectly formed bivalents at the first divisions. The spores are completely good. There is no sign even of the occasional univalents which were observed in the F2 plant raised from the synthetic diploid hybrid. In view of the perfectly regular meiosis found in these tetraploid F2 plants (which, considering their origin from a *P. lonchitis* × *P. setiferum* hybrid, may be regarded as equivalent to *P. aculeatum*) it would be of interest to discover if the two genomes in a haploid plant of *P. aculeatum* would pair together. Such haploids have been produced in *Dryopteris dilatata* (Hoffm.) A.Gray and in *D. filix-mas* (L.) Schott (Manton & Walker, 1954) as well as in *Asplenium* (Bouharmont, 1972a,b) although, as pointed out by Lovis (1977), the potential use of such haploids is limited, since they appear to be a less reliable indicator

of maximum pairing than are wide hybrids. Attempts were made to produce haploid plants of both *P. aculeatum* and *P. braunii*. Water was withheld from gametophytes of both these species in an endeavour to induce apogamous outgrowths, and with the same object in mind prothalli were grown on a nutrient medium rich in sucrose (Whittier & Steeves, 1960). The successful induction of apogamous plants from gametophytes of *P. aculeatum* and *P. braunii* has, unfortunately, yet to be achieved.

Wagner's work on the *Polystichum* species of the western United States (1973) may usefully be compared with the discovery of *P.* × *lonchitiforme* in Ireland and the subsequent raising of a fertile tetraploid generation from this hybrid. Wagner suggested that in North America no less than three putative allotetraploids, *P. californicum*, *P. scopulinum* and *P. kruckebergii*, had all arisen as a result of hybridization between distinct diploid parents, followed by chromosomes doubling. In the case of the first two examples, sterile diploid hybrids were actually found in the field alongside their fertile tetraploid derivatives. The first, *P. californicum*, is a tetraploid species derived from the cross *P. munitum* × *P. dudleyi* (the latter being a very rare coastal Californian endemic, bipinnate and superficially rather similar to *P. setiferum*). There are several localities known where *P. munitum*, *P. dudleyi* and *P. californicum* grow together, mixed with sterile diploid hybrids, that are indistinguishable morphologically from tetraploid *P. californicum* except by the possession of abortive spores, and the two triploid back-cross hybrids, *P. munitum* × *P. californicum* and *P. dudleyi* × *P. californicum*. Both these latter hybrids show 41 paired and 41 single chromosomes at meiosis, and may thus be compared directly with *P.* × *illyricum* (Borbàs) Hahne and *P.* × *bicknellii* (Christ) Hahne, the two European back-cross hybrids involving *P. aculeatum* and showing exactly comparable behaviour. A similar example is the case of *P. scopulinum* which has been shown to be derived from a cross between *P. munitum* and yet another rare diploid in North America, the amphitropical *P. mohrioides*. Yet again a locality was discovered where fertile tetraploids of *P. scopulinum* existed together with sterile diploid hybrids morphologically indistinguishable from them, and with two back-cross triploid hybrids, *P. scopulinum* × *munitum* and *P. scopulinum* × *P. mohrioides*, both of which showed 41 paired and 41 single chromosomes at meiosis. A further North American example (D. Wagner, 1979) involves, not a tetraploid, but the hexaploid, *P. setigerum* (C.Presl) C.Presl, which shows a perfectly regular meiosis with 123 bivalents. David Wagner suggests this hexaploid arose by chromosome doubling from the triploid hybrid, *P. braunii* × *P. munitum*, although in this case the spectacular microcosm of evolution in action, described by W.H. Wagner (1973) with regard to *P. californicum* and *P. scopulinum*, is lacking, as neither of the back-cross hybrids, nor the suggested progenitor, the triploid hybrid *P. braunii* × *P. munitum*, have yet been discovered. The last combination has been synthesized by Sleep (1966) (see Table 2), and it shows a disturbed meiosis with 13 to 28 irregular bivalents. However, that the irregular meiosis observed widely in diploid and triploid hybrids in *Polystichum* is in no way a barrier to the successful nature is shown by W.H. Wagner's discovery of the occurrence of two examples of apparently newly formed fertile allotetraploid species occurring in the wild side by side with morphologically indistinguishable diploid hybrids.

Lovis (1977) has suggested, however, that the tetraploid individuals encountered in the localities described by Wagner (1973) may not be even of relatively recent origin. He assumes that, because of the association of homoeologous chromosomes in the diploid progenitor, newly formed tetraploids will display some degree of meiotic instability, and indeed this appears to be the case in many examples of recently formed auto- and

allopolyploids in flowering plants (Stebbins, 1972). However, the evidence supplied by the F2 generation raised from the wild diploid hybrid *P.* × *lonchitiforme* contradicts this view. The F2 generation shows a perfectly regular meiosis with 82 bivalents, and this is achieved in the course of one generation. Fertility is immediately achieved and does not seem to require the stabilizing effect of generations of selection. If a similar situation pertains in the North American examples, *P. californicum* and *P. scopulinum* may indeed prove to be of relatively recent origin, a suggestion which is supported by the restricted distributions of these two ferns. The distribution of *P. aculeatum* on the other hand, suggests that it is a much older species which has had time to establish itself firmly over most of Europe.

Genetic factors affecting chromosome pairing have been demonstrated to exist in the more intensively studied crop plants, such as hexaploid wheat (Riley & Chapman, 1958; Riley, Chapman & Kimber, 1959; Chapman & Riley, 1970). Indeed, in wheat, by a very elegant series of hybridization experiments, the specific control mechanism has been shown to occur on the long arm of chromosome 5B. The genetic control of chromosome pairing was comprehensively reviewed by Riley & Law (1965), and further illuminated by Dover & Riley (1972) and Riley (1974). The existence of some genetic mechanism controlling chromosome pairing behaviour in ferns has been postulated by several authors (for example, Braithwaite, 1964a; 1986; Sleep, 1966; Sinha & Manton, 1970, Bouharmont, 1977; Lovis, 1977) but has not as yet been directly demonstrated experimentally. In *Polystichum* the tetraploid F2 plants equivalent to *P. aculeatum* and raised by sowing the spores from wild diploid hybrid, *P.* × *lonchitiforme* were immediately fully fertile. It is clear that, if a genetic mechanism which favours bivalent formation (by suppressing meiotic irregularities which will reduce fertility) is indeed present, it has not been evolved subsequent to polyploidy, but must already exist in the genomes of the diploid parental species. It would be of interest to sow spores from Wagner's wild diploid hybrids *P. munitum* × *P. dudleyi* (= sterile *P. californicum*) and *P. munitum* × *P. mohrioides* (= sterile *P. scopulinum*) to see if immediately fertile tetraploid generations having regular diploidized meiosis similar to the fertile F2 from *P.* × *lonchitiforme* can be raised.

Polystichum aculeatum, *P. californicum* and *P. scopulinum* are (because of the incomplete differentiation between the genomes of their diploid progenitors) all of undoubted segmental allopolyploid origin (Stebbins, 1947; 1950; Lovis, 1977). However, *P. aculeatum* (and probably also the two North American tetraploids) has evolved in the direction of genomic allopolyploidy in that it breeds true to its intermediate condition and appears to segregate very little. It is recognizably intermediate between its original parental species in external morphology, ecological preferences and geographical distribution, and it is generally regarded to be genetically isolated from its progenitors through the sterility of the back-cross hybrids, *P.* × *bicknellii* and *P.* × *illyricum*. However, there is evidence that both these hybrids can be partially fertile (Vida & Reichstein, 1975). The production of dyads (or unreduced diplospores) by *P.* × *illyricum* and the raising of hexaploid plants by Vida & Reichstein (1975) have obvious relevance, not only to the origin of *P. setigerum* (D. Wagner, 1979) but to the origin of *P. aculeatum* and the other tetraploids. Large, round spores similar to those recorded in *P.* × *illyricum* and, incidentally in a series of wild and synthetic diploid hybrids in *Asplenium* (Lovis, 1968b; Lovis & Reichstein, 1968a; 1968b; Lovis, 1970), have been observed in the diploid *P.* × *lonchitiforme*, and their mode of formation is currently under investigation. Also relevant to this discussion is the presumed formation and functioning of unreduced gametes that

have been recorded in several different genera during the course of various experimental hybridization programmes (Walker, 1962b; Emmott, 1964; Braithwaite, 1964b; Sleep, 1966; Gibby, 1977). In the present investigation several hybrids of the cross *P. braunii* × *P. acrostichoides* proved to be not triploid, as expected, but tetraploid, and it is suggested that these hybrids resulted from the functioning of an unreduced (i.e. diploid) gamete from *P. acrostichoides* crossing with a normal (diploid) gamete from the tetraploid *P. braunii*. Similar behaviour has been recorded in a wild hybrid *P. braunii* × *P. acrostichoides* collected by Coffin (Thompson & Coffin, 1940) and studied by Morzenti (1962), who found it to be tetraploid. More recently Barrington (1986) has examined other wild examples of *P. braunii* × *P. acrostichoides* which have proved to be uniformly triploid. Although in the instances referred to above unreduced spores can generally been found to result from meiotic irregularities during sporogenesis, Schneller & Rasbach (1984) have reported the production of unreduced spores from spontaneously polyploid tissue on the leaves of the diploid hybrid *Athyrium distentifolium* Tausch ex Opiz × *A. filix-femina* (L.) Roth. Whatever the mechanism of formation of unreduced spores, however, it is clear that the homoeologous pairing so widely observed in diploid hybrids in *Polystichum* presents no barrier to chromosome doubling and to the production of tetraploid progeny, whether in nature or in the laboratory.

This report of the widespread occurrence of homoeologous chromosome pairing in *Polystichum* would be incomplete without some mention of Klekowski's hypothesis that homoeologous chromosomes are not only to be expected, but are generally present in homosporous ferns because of the high levels of polyploidy encountered (Klekowski & Baker, 1966), and that pairing between homoeologous chromosomes allows the release of genetic variability in plants which would otherwise, because of their life-cycle, be expected to produce homozygous sporophytes by habitual self-fertilization (Klekowski, 1973). Klekowski's proposals have been discussed in some detail by Lovis (1977) and by Walker (1979) and it is not necessary to reiterate their arguments here. Although both genetic and cytological evidence of such behaviour has been adduced in *Ceratopteris* (Hickok, 1978), recent research has shown that in many other groups of homosporous pteridophytes genetic evidence is not compatible with Klekowski's proposals, and data obtained from enzyme electrophoresis indicate that homosporous ferns with high chromosome numbers are diploid (e.g. Haufler, 1987).

In conclusion, the origin of tetraploid *P. aculeatum* from two diploid progenitors, *P. lonchitis* and *P. setiferum*, through hybridization and subsequent chromosome doubling is confirmed through re-synthesis from both synthetic and wild diploid hybrids. However, it is clearly shown that chromosome pairing in hybrids in the genus *Polystichum* does not always follow the pattern found extensively in other genera (e.g. *Asplenium*, *Dryopteris*); the significant levels of chromosome pairing discovered in some wide hybrid combinations remain unexplained.

APPENDIX

Origin of plants used in the hybridization programme and wild hybrids, with confirmation of chromosome numbers.

Polystichum taxon	Origin	Collector	Count
P. setiferum	Istanbul, Turkey.	n/a	Diploid
P. setiferum	Grümpeli, Rheinfelden, Aargau, Switzerland	T.Reichstein	Diploid
P. setiferum	Gorge Chauderon, Montreux, Vaud, Switzerland	Sleep, AS/16	Diploid
P. lonchitis	Granite above Bains de Tredos, Pyrenees, Spain	D. Bartley	Diploid
P. lonchitis	Pont-de-Nant, Vaud, Switzerland	Sleep, AS/62	Diploid
P. lonchitis	M-tele Piatra Criului, Romania	n/a	
P. lonchitis	British Columbia, Canada	Stuart Holland	Diploid
P. acrostichoides	McClean Bog, Ithaca, USA	H.G. Baker	Diploid
P. acrostichoides	Vermont, USA	n/a	Diploid
P. munitum	North of Mohles, Oregon, USA	n/a	Diploid
P. aculeatum	Granite scree above Bains de Tredos, Pyrenees, Spain	D. Bartley	Tetraploid
P. aculeatum	Switzerland		
P. braunii	Switzerland		
P. × *lonchitiforme*	Goat's Pass, Glenade, Co. Leitrim, Ireland	Sleep, AS/75/3	Diploid
P. × *wirtgeni*	Val dei Molini, northern side of the Grigna, N. Italy	T. Reichstein TR 471	Triploid
P. × *luerssenii*	Krimml waterfall, Salzburg Austria	Sleep, AS/62/5	Tetraploid

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Membership is open to all interested in ferns and lycophytes. **SUBSCRIPTION RATES** (due on 1st January each year) are Full Personal Members £25, Personal Members not receiving *The Fern Gazette* £21, Student Members £12.50, Subscribing Institutions £42. Family membership in any category is an additional £2.50. Applications for membership should be sent to the Membership Secretary (address above) from whom further details can be obtained. **Airmail** postage for all journals is an extra £10, or for those not receiving *The Fern Gazette* £5. **Standing Order** forms are available from the Membership Secretary and the BPS website.

Back numbers of *The Fern Gazette*, *Pteridologist* and *Bulletin* are available for purchase from
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